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THE TOXIC EFFECTS OF EXCESSIVE DOSES OF IRRADIATED ERGOSTEROL UPON YOUNG ANIMALS.

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IN the earlier stages of these investigations full-grown dogs were employed practically entirely. It was stated in a previous communication (1931) that the smallest dose which caused death in these animals was, weight for weight, about 20 times the therapeutic dose for the human subject. The minimal lethal dose for adult dogs was based upon the potency of the irradiated ergosterol as originally assayed by the manufacturers. This material was designated 10,000 *D*, which meant that its potency was 10,000 times that of cod-liver oil, or 40 times that of the therapeutic material (250 *D*). Later this material was stated by Bills (1931) to be more than 100 times the potency of the therapeutic material (250 *D*), and was therefore designated 10,000 *X* (25,000–30,000 *D*). Therefore the irradiated ergosterol which we employed in our experiments upon adult animals had a potency 2½–3 times greater than the original estimate. In a desire to determine the effect, if any, of a single maximal dose of irradiated ergosterol upon a young dog, a collie of between 3 and 4 months of age, and weighing 4 kilos was selected and given 1 c.c. of irradiated ergosterol (10,000 *X*) by mouth. Three days later the animal appeared bright and active, and apparently normal in every way. The serum calcium was not determined. Two c.c., an admittedly enormous dose, was then given. Extreme weakness and vomiting followed within a few hours, and death occurred within 48 hours after this second dose had been given. The typical signs of acute ergosterol overdosage described in previous papers were found post-mortem. The mucosa of the stomach and intestines showed as intense a reaction as any we have seen. It seemed to us that this animal, which had received 3 c.c. of the sterol in two doses of 1 c.c. and 2 c.c. over a short period, had succumbed more rapidly than one would expect an older dog to do. In the course of earlier experiments we had also been given the general but definite impression that the younger adult dogs were somewhat less resistant than older ones to overdosage.

TABLE I.—*Effect of Irradiated Ergosterol on Pups above 4 months old.*

No. of animal.	Weight before administration in kilos.	Daily dose of irradiated ergosterol 10,000 *X* in c.c.				Number of days from commencement of administration to death.	Serum calcium mgm. per 100 c.c. and symptoms.	Weight at, or shortly before, death in kilos.	Post-mortem findings.
		Total.	Per kilo.	Equivalent of 250 *D* per kilo.	*X* T* per kilo.				
1	2·71	0·3	0·10	10	83	11	Hypercalcæmia (18 mgm.)conjunctivitis, vomiting, diarrhœa, blood in stools	1·7	Flushing of gastric mucosa with small extravasations of blood. Hæmorrhagic areas in lungs.
2	3·77	0·25	0·06	6·6	55	36	Hypercalcæmia (16 mgm.) conjunctivitis and inflammation of lids with loss of eyelashes. Profound emaciation. Vomit- and diarrhœa in early part of experiment	2·77	Stomach and intestines apparently normal.
3	4·76	0·20	0·04	4·0	33	59	Hypercalcæmia (14 mgm.) shortly after commencement followed by recovery to normal	2·31	Stomach and intestines apparently normal.
4	3·69	0·15	0·04	4·0	33	15	Calcium 11 mgm. Loss of weight. No other definite symptoms until 24 hours before death, when vomiting and great weakness, ending in collapse occurred	2·50	Intussusception of lower 12 in. of ileum into colon, invaginated bowel, hæmorrhagic and sections gangrenous.
5	4·11	0·1	0·02	2·0	17	60	Slight rise in serum calcium during first 2 weeks. Fall after this which reached normal 2 weeks later	2·12	Nothing abnormal noted in viscera.

* T = maximal therapeutic dose (0·12 c.c. 250 *D* per kilo).

In order to investigate the apparently greater susceptibility of growing animals to irradiated ergosterol, two groups of pups were selected. The first group comprised 5 animals of about 4 months of age, and weighing from 2·71 to 4·76 kilos (Table I). There were 6 animals in the second group about 2 to 2½ months old, and weighing from ·910 to 1·56 kilos (Table II).

The members of the first group were given ergosterol (10,000 *X*) which for convenience of administration was diluted 1 in 10 with corn oil. The diluted material had a potency, therefore, ten times that of the therapeutic material (250 *D* or 100 *X*). The material was administered by mouth in doses ranging from 3 to 1 c.c. per day. That is, pup No. 1 received 3 c.c., pup No. 2 2·5 c.c., and so on, the dose being reduced by 0·5 c.c. with each successive animal, so that pup No. 5 received 1 c.c. only. Pup No. 1 was the first to die. This animal was, so far as we could judge, 4 to 6 weeks younger than the others. It was also the lightest in weight, and received the largest dose. The first definite symptoms were evident within a few days after the administration was commenced. There was rapid loss in weight, loss of appetite, vomiting, diarrhœa. The serum calcium rose to a high level. There was profound weakness, especially noticeable in the hind-quarters. Apathy and lethargy were pronounced features. There was a purulent conjunctivitis, which kept the lids almost completely closed, and death occurred on the 11th day. The changes in the gastric and intestinal mucosa were much less intense than we had been accustomed to find in adult dogs. A general flushing and a few ecchymoses alone were found. No. 4 was the next to die, on the 15th day. This animal received next to the smallest dose. It weighed 3·69 kilos and was no younger than the rest. Its death came as a surprise, and we did not believe that its death was the result of overdosage. The calcium was scarcely raised above the normal, which fact confirmed us in this conclusion. At post-mortem the stomach showed no abnormality, but the lower 12 in. of the ileum was invaginated into the colon. The imprisoned section of bowel was hæmorrhagic and in parts gangrenous. The intussusception which was certainly the cause of death, was looked upon at the time as probably a fortuitous occurrence, independent of overdosage. We believe now, however, that ergosterol predisposes the young dog to the development of intussusception. Since the condition was observed in this animal a large proportion of young animals have died from this cause following ergosterol overdosage. Of 30 puppies which died as a result of ergosterol overdosage, intussusception was the cause of death in 10. In one other there was definite evidence that an intussusception had existed, but had been released, and in others the lower ileum was strongly contracted to a small cord, while the adjoining colon was widely dilated. The two sections of bowel in this instance were each in a state which must have been conducive to the development of intussusception. In three of the instances the intussusception was double. That is, the ileum had passed for a distance of from 6 to 12 in. within the colon and the latter had again become invaginated into itself. In one animal intussusception was the cause of death after a single large dose of irradiated ergosterol had been injected intravenously. These effects of activated ergosterol upon the motor mechanisms of the bowel cannot be dealt with further in the present paper, but it may be said that of the large number of young dogs of approximately the same age

TABLE II.—*Effect of Irradiated Ergosterol on Pups 2–2½ months old.*

No. of animals.	Weight before administration in kilos.	Daily dose of irradiated ergosterol 10,000 *X* in c.c.				Number of days from commencement of administration to death.	Serum calcium mgm. per 100 c.c. and symptoms.	Weight at death in kilos.	Post-mortem findings.
		Total.	Per kilo.	Equivalent of 250 *D* per kilo.	*X* T* per kilo.				
100	1·18	0·35	0·29	29·0	240	8	Hypercalcæmia (20·8 mgm.), vomiting, great weakness, especially of hind quarters, blood highly concentrated	0·80	Deep flushing of gastric mucosa, hæmorrhagic patches in lungs, intussusception of 3 in. of lower ileum into colon, bone-marrow hæmorrhagic.
101	1·25	0·30	0·24	24·0	200	11	Hypercalcæmia (22·6 mgm.), emaciation, weakness leading to complete prostration	0·87	Ileum contracted, a thin cord, cæcum widely dilated, conditions appear preparatory for development of intussusception, lungs show hæmorrhagic areas 1 cm. square, bone-marrow hæmorrhagic.
102	1·25	0·25	0·2	20·0	166	12	Hypercalcæmia probably present, but calcium determinations not made. Symptoms similar to preceding	0·90	Intussusception of 3 in. of lower ileum. Gastric mucosa shows flushing only.
103	1·32	0·20	0·14	14·0	116	14	Hypercalcæmia (24·0 mgm.). Symptoms similar to those of preceding animals	0·90	Gastric mucosa injected, a few ecchymotic areas. Bowel filled with dark brown fluid. Lungs show well-defined hæmorrhagic areas. A section of ileum about 3 in. long deeply congested and showing a hæmorrhagic area ½ in. long. The mesentery of this section of the ileum injected, vessels dilated. Ileum on either side of this area is quite pale and normal in appearance.
104	0·91	0·15	0·16	16·0	136	6	Diarrhœa, weakness, particularly of hind-quarters, comatose last few hours of life	0·78	Lungs hæmorrhagic, stomach mucosa colour normal, bone-marrow hæmorrhagic.
105	1·56	0·10	0·06	6·0	50	13	Hypercalcæmia (19·4 mgm.)	1·03	No hæmorrhages in lungs. Stomach bright red in pyloric region, duodenum pale, bone-marrow hæmorrhagic, ileum firmly contracted, in sections separated by dilated pouches, but there is no intussusception.

* T = maximal therapeutic dose.

employed as controls, only 2 died. The cause of death in these animals was not determined, but in neither was intussusception found. There is no reason, moreover, to believe that intussusception is a common occurrence in young dogs under laboratory conditions. We must therefore conclude from these results that in young dogs irradiated ergosterol induces a disturbance in the motor mechanisms of the bowel which predisposes to the condition. Intussusception is an effect of the administration which usually supervenes early and causes death before the other signs of overdosage are apparent.

Of the remaining three pups of Group I all were alive one month after the commencement of the ergosterol administration. In all, the serum calcium was elevated and all had lost a large proportion of their body-weight. The rapid loss in weight was especially noticeable in pups Nos. 3 and 5. The latter animal had received only 0·10 c.c. of irradiated ergosterol (10,000 *X*) daily. At the beginning of the experiment its weight was 4·11 kilos. Two weeks later, even upon a casual observation, its extreme emaciation was very noticeable. Its weight shortly before death was 2·12 kilos, which represented a loss of nearly 50 per cent. of the body-weight. The three other animals showed a loss of weight to a somewhat less degree. It is to be remembered that all were young animals, and consequently the figures do not represent the full effect which irradiated ergosterol exerts upon the body-weight. These animals, if untreated, would have been expected to add from 1 to 2 kilos to their weights during periods of similar duration.

The second group of 6 animals (Nos. 100–105), though considerably younger, were given similar doses of irradiated ergosterol. The undiluted material (10,000 *X*) was employed, and the bulk of each dose was correspondingly smaller. Thus pup No. 100 received 0·35 c.c., pup 101 0·3 c.c., and so on. Pup 105 received only 0·1 c.c. of this material. All these animals died within 2 weeks. The results are shown in Table II.

Evidently in these two groups of animals the minimal lethal dose of irradiated ergosterol had not been reached. In order therefore to arrive at some idea of what this might be, it was decided to give two large doses in succession to each animal in a group and note the survival periods. A large dose of irradiated ergosterol is apparently either not quickly absorbed, but remains in the gastro-intestinal tract for some days, or continues to circulate in the blood and exert its effects for a considerable period after its administration. It was therefore considered justifiable, in order to arrive at a tentative estimate of the minimal daily dose of irradiated ergosterol that would produce signs of overdosage, to take the quantity of the total dose in c.c. administered to these animals, and divide it by the survival time in days. Four young pups about 2 months old were given 0·5, 0·4, 0·3 and 0·2 c.c. respectively of viosterol (10,000 *X*) by mouth on two successive days. The results are shown in Table III.

In the foregoing series the animals were fed stock laboratory diets consisting of milk and bread or dog biscuit and a small quantity of meat. No vitamins were added. Although puppies thrive and grow upon this diet, it was thought possible that it was not adequate, and that a more liberal diet including the necessary vitamins might enable the animals to resist better the effects of ergosterol overdosage. In the next series particular attention was therefore

TABLE III.—*Effect of Two Large Doses of Irradiated Ergosterol.*

Pup No.	Weight in kilos.	Number of days after first dose till death.	Total dose (10,000 *X*) in c.c.	Calculated dose per day = total dose ÷ days of survival.	Calculated dose per kilo per day in c.c.	Viosterol (250 *D*) equivalent per kilo per day in c.c.	*X* T.*
110	1·46	7·5	1·0	0·13	0·08	8·0	66
111	1·39	8	0·8	0·10	0·07	7·0	58
112	0·91	5	0·6	0·12	0·13	13·0	108
113	1·6	8	0·4	0·05	0·03	3·0	25

* T = maximal therapeutic dose.

paid to the diet in order to ensure that it did not lack any requisite for health and normal growth. Six police pups of the same litter, about 2 months of age, were chosen for this experiment. They were fed whole milk, dog biscuit and 20 gm. of meat daily. Vitamins B and C were supplied by adding suitable amounts of bemax and tomato-juice. Upon admission to the laboratory they were strong, well-nourished animals, full of vigour and apparently in perfect condition. They were placed upon the above diet, and kept under observation for two weeks after admission. Some weight was lost during the first week, but a gain was shown at the end of the second week. Irradiated ergosterol administration was then commenced. The dosage ranged from 0·05 c.c. to 0·005 c.c. of 10,000 *X* per kilo, as shown in Table IV. Four of the animals had lost weight at the end of 10 days of treatment, and all weighed less at the end of 3 weeks. The animal receiving the largest dose died on the 18th day The one receiving the smallest dose survived for 2 months ; a slight gain in weight occurred during the first 5 weeks, but from this time on there was a steady decline. The dose received by this animal was only four times the maximal therapeutic dose. Though caution must always be exercised in drawing conclusions applicable to man from experiments upon animals, these results certainly suggest that the administration of irradiated ergosterol to infants in amounts not exceeding so very greatly those at present recommended may be fraught with danger.

In animals receiving comparatively small, though still toxic, doses of irradiated ergosterol over long periods hypercalcæmia does not always occur. For this reason we are forced to fall back upon other less reliable criteria of overdosage, such as loss of body-weight, lowered vitality and survival period. Nevertheless, though snuffles or pneumonia occasionally caused the death of the control animals, the deterioration of the animals which invariably followed the administration of ergosterol leaves little room for doubt, when one considers the number of animals employed, that overdosage was the cause of death. Recently we have been following the calcium excretion of animals so treated, with the view of obtaining a more precise index of overdosage. We have found so far that in doses comparable with those given to the preceding groups a very definite increase in total calcium excretion occurs. The results of these experiments will be reported shortly.

The final series of growing animals treated with irradiated ergosterol were somewhat older than those in the preceding groups. These animals, 8 in number, were about 3 months of age, vigorous and apparently in good health

TABLE IV.—*Effect of Irradiated Ergosterol on Pups about 8 weeks old, fed on a Diet containing Adequate Vitamins B and C.*

Puppy No.	Weight in kilos.								Daily dose per kilo 10,000 *X* in c.c. from Jan. 26, 1931.	Daily dose in equivalent of 250 *D* in c.c.	*X* T.*
	Jan. 26, 1931.	Feb. 5.	Feb. 14.	Mar. 2.	Mar. 9.	Mar. 16.	Mar. 23.	Mar. 30.			
1	2·39	2·20	Died Feb. 13. Wt. 1·60	..	..	..	..	..	0·05	5	42
2	2·53	2·38	2·33	2·5	Died Mar. 9. Wt. 1·75	..	..	..	0·04	4	33
3	2·24	2·19	Died Feb. 14. Wt. 1·62	..	..	..	..	..	0·03	3	25
4	2·32	2·25	2·30	2·61	2·52	Died Mar. 14. Wt. 1·68	..	..	0·02	2	17
5	2·40	2·45	2·21	1·89	Died Mar. 3. Wt. 1·6	..	..	..	0·01	1	8
6	2·40	2·46	2·24	2·49	2·30	2·25	2·04	Died Mar. 27. Wt. 1·32	0·005	0·5	4

* T = maximal therapeutic dose.

at the commencement of the experiment. They were fed upon a diet of whole milk, bread, meat and the necessary vitamins. The animals were divided into two groups of 4 each. One group received irradiated ergosterol in doses ranging from 0·3 to 0·7 c.c. of 10,000 *X* per kilo daily. The other group served as controls. Every precaution was taken to keep the animals in good condition. They were removed from all other animals in the laboratory and placed in a separate room in another wing of the laboratory and were allowed to roam in the open-air for several hours daily.

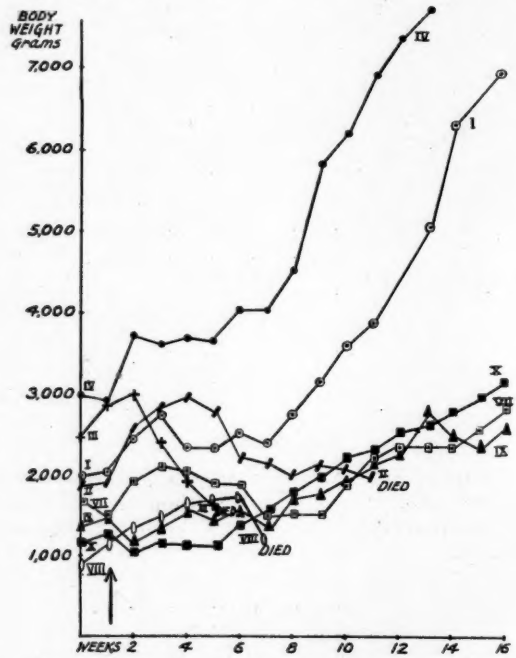

FIG. 1.—Growth-curve of pups of Table V. Animals II, III, VIII and X treated with irradiated ergosterol. Arrow indicates time administration was commenced. Nos. I, IV, VII and IX are the control animals.

The ergosterol administration was not commenced until all animals had become accustomed to their new surroundings and were gaining in weight. The experiment extended over a period of 25 weeks. Of the 4 treated animals, those receiving the three largest doses died in from 4 to 10 weeks. The animal receiving the smallest dose survived and was alive at the end of 6 months. All the control animals survived for the same length of time, and were then used for other experiments. The treated animals received irradiated ergosterol (10,000 *X*) once daily. The dosage and survival times of the four treated animals are given in Table V. The growth-curves of the 8 animals are shown in Fig. 1.

TABLE V.—*Effect of Irradiated Ergosterol on Pups about 3 months old, fed on a Diet containing Adequate Vitamins B and C.*

Pup No.	Weight in kilos.	Survival time, weeks.	Dose 10,000 *X* per kilo in c.c.	Equivalent in 250 *D* per kilo in c.c.	*X* T.*
II	2·29	10	0·07	7·0	58
III	2·27	4	0·06	6·0	50
VIII	1·20	6	0·04	4·0	35
X	1·60	Until conclusion of experiment	0·03	3·0	25

* T = maximal therapeutic dose.

It will be observed that there was not complete correspondence between the size of the doses and the survival periods. Of those which died, pup No. II, though it received the largest dose, lived the longest. This, however, is not surprising, since we have found that animals varied rather widely in their resistance to overdosage. The fourth animal (pup No. X) also tolerated a dose considerably larger than some pups in other series, which may be accounted for by the fact that the animals in this last series were somewhat older than the majority of those in the earlier experiments.

All these animals, including the controls, showed a flattening or decline in the growth-curve some time between the second and ninth weeks (middle of June to end of July) of the experiment. This was probably due in large part at any rate, to the intensely hot weather which occurred during this time.

SUMMARY.

Experiments upon the effects of irradiated ergosterol on young dogs are described. The doses employed were on the whole smaller than those used in previous experiments upon adult animals, and the administration was extended over longer periods.

The resistance of individual animals to the toxic effects of irradiated ergosterol varies considerably. In some animals a daily dose less than ten times the maximal infant dose, which is taken to be 0·12 c.c. of 250 *D* per kilo, proved definitely toxic On the other hand, one animal remained apparently unaffected by a daily dose which was twenty-five times the maximal therapeutic dose.

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STUDIES ON *CLOSTRIDIUM CHAUVŒI*. I: THE ANALYSIS OF THE "H" AND "O" ANTIGENS OF *C. CHAUVŒI*.

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THE extensive researches on the ætiology and prophylaxis of blackleg as it occurs in cattle and sheep have recently been critically reviewed by Bosworth (1930), and the balance of evidence indicates *C. chauvœi* as the specific ætiological factor. He concludes that blackleg is a specific disease caused by *C. chauvœi*.

Blackleg in the sheep usually occurs as a result of a wound infection, whereas in cattle no such history exists, the disease making a sudden and as yet unaccountable onset. This fact, together with the statement first made by German workers, that blackleg in cattle and blackleg in sheep, although primarily *C. chauvœi* infections, do not occur in the same district, has led to extensive comparative studies of strains isolated from cattle and sheep. These studies, however, have failed to show any fundamental differences between strains either in morphology, culture, or serological relationships.

The intimate antigenic structure of *C. chauvœi* strains as studied by the agglutination reaction has been explored by few workers, probably on account of the attendant auto-agglutination experienced with these organisms. The researches of Zeissler (1924), Zeller (1927) and Jungherr (1928), however, on this point have failed to reveal any serological variation among strains of bovine and ovine origin.

With the introduction of the method of "qualitative receptor analysis" of Weil and Felix (1917), first as applied to the aërobes, and recently extended to the spore-bearing anaërobes *C. œdematis maligni* and *C. tetani* by Felix and Robertson (1928), the serological technique primarily used for the study of the agglutination reaction among the spore-bearing anaërobes is open to criticism. It would appear that the reactions observed have been due in most part to the labile "H" antigen, and that the reaction of the "O" antigen has been overlooked. It has been shown further, by Robertson and Felix (1930), that with *C. œdematis maligni*, as with the aërobes, the "O" antigen appears to be the sole important factor in the production of antibacterial mechanisms in the animal body.

The present investigation, therefore, is concerned with the application of the method of "qualitative receptor analysis" to *C. chauvœi* strains of bovine and ovine origin, collected from various parts of the world, with a view to establishing their "O" and "H" relationships. In a later paper the "O" antigen is examined as a factor in the production of active immunity.

SOURCE OF CULTURES USED.

Table I gives details as to source and the laboratory reference numbers of cultures used. By request, all strains were received in meat broth subculture, generally in duplicate. Cultures were stored sealed in chopped meat broth and alkaline egg after 48 hours' anaërobic incubation at 37° C.

TABLE I.—*Strains of* B. chauvœi *investigated.*

Laboratory reference number.	Strain.	Isolated from—	Country of origin.	Received from—
287	Bovine	Blackleg in cattle	England	Nat. Coll. of Type Cultures.
C	,,	Ditto	,,	Ditto.
M	,,	,,	,,	,,
H	,,	,,	,,	,,
G1	,,	Blackleg muscle from heifer	,,	Prof. S. H. Gaiger, Dept. Vet. Pathol. Liverpool.
B1	,,	Blackleg ?	,,	Mr. T. J. Bosworth, Inst. Animal Pathol. Cambridge.
M4	Ovine	Parturient gangrene in ewe	,,	Mr. A. D. McEwen, South Eastern Agric. College, Wye.
M7	,,	Spontaneous blackquarter in ewe	,,	Ditto
A9	,,	Ewe dead of blood-poisoning	S. Africa	Dr. P. J. Du Toit, Onderstepoort, S. Africa.
A10	Bovine	Blackquarter muscle	,,	Ditto.
3265	,,	Blackquarter in calf	Australia	Nat. Coll. of Type Cultures.
3266	Ovine	Blackquarter in ewe	,,	Ditto.
16	Bovine	Blackquarter in cow	U.S.A.	Dr. H. Marsh, Vet. Research Lab., Bozeman, Montana, U.S.A.
19	Ovine	Blackquarter in sheep	,,	Ditto

MORPHOLOGICAL AND CULTURAL CHARACTERISTICS.

The morphological and cultural characteristics of *C. chauvœi* as described by McEwen (1926) and Robertson (1929) have been taken as identifying typical *C. chauvœi.* The following points of variance have, however, been recorded: (*a*) Spore-formation varies greatly with the strain. Certain strains, which

have been maintained in subculture for at least five years, form spores with the greatest difficulty even on the most favourable media. From this state there is a gradation in spore-production, a maximum sporulation of approximately 75 per cent. being produced when incubated 48 hours on Dorset egg medium. After rapid and repeated subculture in meat broth for a period of from two to three months the ability to sporulate appears to diminish, and reversion to old sealed subcultures is necessary to maintain high spore-production. (*b*) McEwen failed to grow *C. chauvœi* on plain glucose broth, and devised the diffusion shell method to obtain large volume growths. In the present work 1 per cent. glucose tryptic digest broth pH 7·8 without the addition of any animal tissue gave satisfactory results after 14 to 16 hours' anaërobic incubation. A 10 per cent. inoculum of meat broth was used for quantities of glucose broth up to 20 c.c. For larger volumes up to 1 litre, a 10 per cent. inoculum was used from a 7- to 14-hour glucose broth culture. (*c*) A characteristic of certain *C. chauvœi* strains is that on storage on artificial media for prolonged periods, there appears to be a complete loss of virulence for guinea-pigs and mice.

PREPARATION OF RABBIT IMMUNE SERA.

Suspensions for rabbit inoculations were prepared from 14- to 16-hour growths in 1 per cent. glucose broth used in quantities from 50 to 1000 c.c., centrifuged, twice washed in distilled water and resuspended in distilled water to an opacity ten times stronger than B.W. & Co.'s standard opacity tube, No. 10. They were further treated differently as described below, and a 10 per cent. solution of sodium chloride was finally added to adjust the salt concentration to 0·85 per cent. No preservative was added to the suspension.

For the preparation of "H" immune sera, suspensions were heated for 1 hour at 60° C. Rabbits received 4 to 6 intravenous inoculations, given on alternate days, in increasing doses from 0·05 to 3·0 c.c., the smaller amounts being diluted with 0·85 per cent. saline to make a total volume of 2·0 c.c. Seven days after the last inoculation rabbits were bled.

For the preparation of "O" immune sera suspensions were heated for 2 hours at 100° C. Rabbits received from eight to ten intravenous inoculations, in increasing doses from 0·5 c.c. to 5·0 c.c., reaching the maximum on the sixth inoculation. The first six inoculations were given on alternate days. Ten days later rabbits were bled, and inoculations recommenced as previously, final bleedings being taken 8 to 10 days after the last inoculation.

All rabbits, previous to inoculation, were bled and carefully tested for the presence of normal agglutinins. None of the sera tested showed agglutination in a dilution of 1 in 10. The normal serum from each rabbit was retained for use as controls with immune sera. Bleedings were made from the jugular vein. Sera were stored at $\pm$ 4° C. without the addition of any preservative.

METHODS USED IN THE AGGLUTINATION TEST.

The preparation of stable suspensions of the spore-bearing anaërobes is a fundamental difficulty in agglutination tests with these organisms. Most strains of *C. chauvœi*, suspended in normal saline, show auto-agglutination

in a period of from 15 minutes to 3 hours within temperatures ranging from 14° to 56° C. The degree of agglutination varies with the same strain on different days, from complete precipitation, to the formation of small loose floccules to be observed only with the hand lens. While certain strains are regularly more stable than others, occasionally giving readable results after 6 to 12 hours, no reliance could be placed on such suspensions.

To overcome this difficulty various methods were attempted, viz. the use of reduced saline concentrations as used by Arkwright (1921) for rough variants of the Salmonellas, the use of hypertonic saline, the surface tension depressants used by Mellon *et al.* (1924) for diphtheroids, Northrop and De Kruif's (1923) use of buffered solutions in attempts to raise surface potential, and White's (1926) alcohol extraction method. No reliable results were obtained. It was further attempted to obtain stable suspensions by altering growth conditions, viz. by the addition of calcium carbonate to neutralize the acid production during growth in glucose broth, the use of sugar-free liquid media, variation in the period of incubation and the use of surface growths. All of these methods proved unsatisfactory.

The following methods were finally employed:

(*a*) For the examination of the "O" antigen: Suspensions heated at 100° C. for 2 hours, as used for the preparation of "O" immune sera, were found to be stable over a wide range of saline concentrations. It was thought, however, that the prolonged heating might, in part, destroy the sensitiveness of the "O" antigen. Table II illustrates the effect of varying periods of exposure to 100° C. on the stability of a suspension and on the agglutination titre.

TABLE II.—*Effect of Heating at 100° C. on Stability and Agglutinability of Suspensions.*

	Serum dilution.	Agglutination with strain G1. Heated at 100° C. for— 5 minutes.	15 minutes.	30 minutes.	1 hour.	2 hours.	3 hours.	Unheated control.
Rabbit immune "O" serum 19 III (Strain 3266)	1 : 100	+++	+++	+++	+++	+++	+++	+++
	200	+++	+++	+++	+++	+++	+++	+++
	500	++	++	++	++	++	+±	+++
	1000	+	+	+	+	+	+	+++
	2000	(±)	(±)	(±)	(±)	(±)	(±)	+++
	5000	−	−	−	−	−	−	+++
Normal rabbit serum 19 I	1 : 50	−	−	−	−	−	−	+++
	200	−	−	−	−	−	−	+++
	Per cent. NaCl.							
Sodium chloride controls	0·04	−	−	−	−	−	−	+
	0·21	−	−	−	−	−	−	+++
	0·42	−	−	−	−	−	−	+++
	0·85	−	−	−	−	−	−	+++
	1·70	−	−	−	−	−	−	+++
	3·40	−	−	−	−	−	−	+++
	6·80	−	−	−	−	−	−	+++

+++ to ± = varying degrees of agglutination observed by the naked eye.
(±) = trace of agglutination estimated by the hand lens.
Suspensions adjusted to opacity 10 × 5 (B. W. & Co. tubes) in distilled water. Readings taken after 4 hours' incubation at 45° C.

Heating for 5 minutes at 100° C. is as effective as 2 hours' exposure, and the agglutination titre shows no change throughout the range. It is seen further, that the unheated suspension was completely precipitated throughout the whole range of saline concentrations. Varying the density of the suspension to be heated in a range from opacity 5 to one ten times greater than opacity 10 had no appreciable effect on the final " O " agglutination titre. Suspensions, therefore, to be used for " O " agglutination were grown in 1 per cent. glucose broth, incubated 14 to 16 hours, centrifuged and twice washed in distilled water, resuspended in distilled water to the required opacity, and heated for 30 minutes to 1 hour at 100° C. The salt concentration was adjusted to 0·42 per cent. with 10 per cent. sodium chloride. Suspensions thus prepared, stored at ± 4° C. without preservative, remained sensitive for at least 6 weeks. Agglutination tests were conducted in 0·42 per cent. saline, and readings were made after 4 hours' incubation at 45° C., and again after standing 18 to 24 hours at room temperature.

(*b*) For the examination of the " H " antigen: In attempts to prepare flagellar suspensions it was found that shaking had a good effect on stability, and the following method for the preparation of " H " suspensions was adopted. Centrifuged growths from 14 to 16 hours' glucose broth cultures were once washed in normal saline, centrifuged and resuspended in normal saline to an opacity ten times stronger than tube No. 10 on the B.W. & Co. series. Quantities of 5 c.c. were shaken in thick-walled glass tubes of approximately 10 c.c. capacity for 1 hour at a speed of 250 to 300 complete shakes per minute. One to two drops of octyl alcohol were added to prevent foaming. Suspensions were then centrifuged for 10 minutes at 2000 to 3000 revs. per minute, the supernatant carefully removed and used as " H " suspension. Microscopic examination of such suspensions showed them to be composed of masses of flagella in which were embedded numerous bacillary bodies. Stored at ± 4° C. without preservative they remained sensitive for at least 48 hours. Their keeping properties have not yet been examined further. Test-tubes when put up with " H " suspensions were apparently water-clear, but in the presence of " H " sera large loose floccules rapidly formed, either visible to the naked eye or with the use of a hand lens. Readings were made after 2 hours' incubation at 37° C., and again after varying periods at room temperature, up to 20 hours.

All agglutination tests were controlled throughout with normal rabbit sera, and with stability tests in a range of saline concentrations.

The sensitiveness of both types of suspensions varied with the same strain, the degree of agglutination in the lower dilutions being principally affected, and the actual end-titre varying only within the limits of the two final dilutions.

In the study of " H " agglutination all sera were previously absorbed with suspensions heated for 2 hours at 100° C., and controls were included to show that " O " agglutinins had been completely removed.

THE " O " ANTIGEN.

In the analysis of " O " antigens the production of agglutinins in rabbits, direct and cross agglutination and absorption of agglutinins were considered.

"O" immune rabbit sera were prepared from representative strains of bovine and ovine origin from each country from which strains were available.

From a study of eight bovine and five ovine strains thus examined, it was found that each "O" immune serum agglutinated all strains to approximately the same titre. Table III shows the results of a cross agglutination test with six sera and eight strains.

TABLE III.—*Cross "O" Agglutination Test.*

Rabbit "O" immune sera prepared from—	Titre of "O" agglutination with strains.							
	English.		South African.		Australian.		American.	
	Bovine. G1.	Ovine. M7.	Bovine. A10.	Ovine. A9.	Bovine. 3265.	Ovine. 3266.	Bovine. 16.	Ovine. 19.
G1	1000	1000	500	1000	1000	1000	1000	1000
M7	1000	500	500	500	1000	1000	1000	1000
A10	2000	2000	1000	2000	2000	2000	2000	2000
A9	1000	1000	1000	1000	1000	1000	1000	1000
3265	2000	2000	1000	2000	2000	2000	2000	2000
3266	2000	1000	1000	1000	2000	1000	2000	2000
Controls.								
Normal rabbit serum 17 I	0	0	0	0	0	0	0	0
Normal rabbit serum 19 I	0	0	0	0	0	0	0	0
0·85% NaCl	0	0	0	0	0	0	0	0

Titre 0 = a negative result in a dilution 1 : 50.
Suspensions of strains used for agglutination steamed 1 hour at 100° C., in distilled water.
Reading taken after 18 hours (4 hours at 45° C., then at room temperature).

TABLE IV.—*Cross Absorption of "O" Agglutinins.*

"O" serum 11 II (Strain M7) absorbed in dilution 1 : 50 with strains.

Agglutination with strains.		English.			South African.		Australian.		American.		"O" serum 11 II, unabsorbed.
		Ovine.		Bovine.	Ovine.	Bovine.	Ovine.	Bovine.	Ovine.	Bovine.	
		M 7.	M 7.	G.	A 9.	A 10.	3266.	3265.	19.	16.	
	Absorbing opacity.	10.	10 × 2.	10 × 2.	10 × 2.	10 × 2.	10 × 2.	10 × 2.	10 × 2.	10 × 2.	
M7	Titre of agglutination with strain M7	50	0	0	0	0	50	0	0	0	500

"O" serum 21 III (Strain A10) absorbed in dilution 1 : 250 with strains.

		South African.		English.		Australian.		American.		"O" serum 21 III, unabsorbed.
		Bovine.	Ovine.	Bovine.	Ovine.	Bovine.	Ovine.	Bovine.	Ovine.	
		A 10.	A 10.	G 1.	M 7.	3265.	3266.	16.	19.	
	Absorbing opacity.	10.	10 × 5.	10 × 5.	10 × 5.	10 × 5.	10 × 5.	10 × 5.	10 × 5.	
A10	Titre of agglutination with strain A10	1000	500	250	250	250	250	250	250	5000

Titre 0 = a negative result in dilution 1 : 50.
Suspensions for absorption twice washed in distilled water; heated 1 hour at 100° C. in distilled water in opacity 10 × 10; diluted as required (*vide* Table) and centrifuged. The serum dilution in 0·42 per cent. NaCl was added to the centrifuged deposits using 1 c.c. serum dilution per 1 c.c. original suspension; incubated 1½ hours at 37° C., and centrifuged.
Reading taken after 24 hours (4 hours at 45° C., then at room temperature).

Complete cross-absorption tests were not performed with all sera and strains. Numerous such experiments, however, gave the uniform result that each strain absorbed the " O " agglutinins from any given serum to the same degree. Table IV illustrates such absorption tests with two sera and eight strains.

THE " H " ANTIGEN.

" H " immune sera were prepared from the strains used for the production of " O " sera, and were tested for cross-agglutination, but no absorption tests of " H " agglutinins were carried out. Table V gives the result of such a test with one ovine and one bovine serum.

TABLE V.—*Cross " H " Agglutination.*

Pure "H" serum. (Sera absorbed in dilution 1 in 500 with suspensions of their homologous strains heated for 2 hours at 100° C.	Serum dilution.	Flagellar suspensions of strains. Australian. Ovine. 3266.	Australian. Bovine. 3265.	S. African. Bovine. A10.	S. African. Ovine. A9.	American. Bovine. 16.	American. Ovine. 19.	English. Bovine. G1.	English. Ovine. M7.	English. Ovine. M4.
Rabbit immune serum 20 III (ovine strain 3266)	1 : 1,000	+±	+±	++	+±	++±	++±	++	−	−
	5,000	+	+±	+±	+±	++	++±	++	−	−
	10,000	+	+	+	+	+±	+±	+±	−	−
	20,000	±	+	±	+	+	+	+	−	−
	50,000	±	(±)	(±)	(±)	±	(±)	±	−	−
	100,000	−	−	−	−	−	−	−	−	−
Rabbit immune serum 22 III (bovine strain A 10)	1 : 1,000	+±	+±	++	+±	+±	++±	++	−	−
	5,000	+	+±	+±	+±	+±	++±	+±	−	−
	10,000	±	+	+	+	+	+±	+	−	−
	20,000	(±)	+	+	(±)	±	±	±	−	−
	50,000	−	−	(±)	−	(±)	(±)	(±)	−	−
	100,000	−	−	−	−	−	−	−	−	−
Controls.										
Normal rabbit serum 23 I	1 : 500	−	−	−	−	−	−	−	−	−
	2,000	−	−	−	−	−	−	−	−	−
0·85% NaCl	..	−	−	−	−	−	−	−	−	−

All agglutinations of pure floccular " H " type.

Controls with steamed suspensions were performed, but are not included in the table; they showed that " O " agglutinins had been completely removed from the two sera.

Reading taken after 2 hours' incubation at 37° C.

It is seen from Table V that a common " H " antigen is distributed equally among all the strains tested, with the exception of the English ovine strains M7 and M4. These two strains possess a specific " H " antigen in common. Further investigation, however, showed that there is a certain overlapping between the two groups. When the two sera used in Table V were absorbed in dilutions lower than those used in this experiment, the presence of a common " H " agglutinin could be demonstrated in dilutions up to 1 in 200 to 1 in 500; *vice versa*, sera prepared against the two English ovine strains M7 and M4 contained, in addition to their own specific " H " agglutinins, low titre agglutinins against the other strains tested. This relationship is shown in Table VI.

TABLE VI.—*Specific "H" Antigen of English Ovine Strains.*

Pure "H" sera. (Sera absorbed in dilution 1 in 50 with suspensions of their respective homologous strains heated for 2 hours at 100° C.)	Titre of "H" agglutination with flagellar suspensions of strains.			
	English.			Australian.
	Ovine. M7.	Ovine. M4.	Bovine. C.	Ovine. 3266.
23 II (Strain M 7)	10,000	5000	500	500
27 II (Strain M 4)	10,000	5000	500	500
5 IV (Strain C)	200	200	50,000	50,000
20 III (Strain 3266)	200	200	20,000	20,000
Controls.				
Normal rabbit serum 23 I	0	0	0	0
Normal rabbit serum 5 I	0	0	0	0

Titre O = a negative result in dilution 1 : 50.

Controls with steamed suspensions are not included in the Table; they showed that "O" agglutinins had been completely absorbed from all sera.

Reading taken after 20 hours (2 hours at 37° C., and then at room temperature).

DISCUSSION.

In a recent paper, Roberts (1931) reports on the serological analysis of four ovine and three bovine strains of *C. chauvœi* of English origin. He finds an "O" antigen common to all strains, and states "that the 'H' component is specific to each type, ovine and bovine." From cross "H" agglutination tests he found that bovine and ovine sera agglutinate "heterologous suspensions to a low titre," but it is not stated whether this agglutination resembled the typical "H" floccular type or was purely of the "O" granular type. In the present work it has been found that separate suspensions are required for a differentiation of "O" and "H" agglutinins, and further, to detect low titres of "H" agglutinins it is necessary to use "H" sera from which the "O" antibody has been completely absorbed. When suspensions of *C. chauvœi* are tested with sera containing both "H" and "O" antibodies, the type of agglutination is difficult to determine accurately.

Felix and Robertson (1928), in their studies on *C. œdematis maligni* and *C. tetani*, established a considerable overlapping of the "O" antigen between different strains of the two species. In later studies, however, on these organisms and on *C. welchii*, wide diversity of the "O" antigen was found in members of these three species (Felix and Robertson, communicated). The uniformity of "O" antigen exhibited by all strains of *C. chauvœi* tested would appear therefore to put this species in a unique position among the spore-bearing anaërobes.

The practical significance of this unity in "O" antigen in the prophylaxis of blackleg in cattle and sheep would appear to lie in the value of this antigen as a factor in the production of immunity. McEwen (1926), Green (1929), and Bosworth (1930), have given evidence of the prophylactic value of washed suspensions of *C. chauvœi*, but their work does not attempt to differentiate between the relative importance of the "H" and "O" components. Further,

their experiments do not include an examination of bovine and ovine strains in the production of cross-immunity.

With *C. œdematis maligni* the value of " O " antigen in the production of a pure anti " O " serum of high prophylactic potency has been demonstrated by Robertson and Felix (1930). It remains, therefore, to determine the value of pure " O " antigen in the production of active and passive immunity to *C. chauvœi*, and to establish the relationship of bovine and ovine strains in this respect by *in vivo* experiment (*see* Henderson, 1932).

SUMMARY.

1. *C. chauvœi* strains of bovine and ovine origin possess a labile " H " antigen and a stable " O " antigen.

2. The use of separate flagellar (" H ") suspensions and steamed (" O ") suspensions is recommended for the accurate differentiation of these antigens.

3. The " O " antigen is uniform in all ovine and bovine strains tested from England, South Africa, Australia and the United States of America.

4. The " H " antigen appears to be complex:

(*a*) A common " H " antigen is distributed equally among all strains tested, with the exception of the English ovine strains.

(*b*) English ovine strains possess a specific " H " antigen.

(*c*) An overlapping component of the " H " antigen is shared by members of these two sub-groups.

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STUDIES ON *CLOSTRIDIUM CHAUVŒI*. II: ACTIVE IMMUNIZATION WITH PURE "O" ANTIGEN.

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In a previous paper (Henderson, 1932) the identity of "O" antigen in ovine and bovine strains of *C. chauvœi* from various parts of the world was established by *in vitro* methods, and the possible value of such an antigen in the prophylaxis of blackleg was indicated.

The present work is a consideration of the "O" antigen as an agent in the production of active immunity against *C. chauvœi* infection, and a further examination of its identity in strains of bovine and ovine origin by *in vivo* experiment.

PRELIMINARY INFECTION EXPERIMENTS.

The experimental animals used were guinea-pigs and mice. A fundamental difficulty in the use of guinea-pigs is known to be their great variation in powers of resistance to infection with whole culture of *C. chauvœi*. In attempts to secure a reliable infection, whole cultures of varying ages from different liquid media were tested, with unsatisfactory results. It was thought that the growth products of *C. chauvœi* might not be reliable activators for the initiation of infection, and later experiments have shown this to be one of the possible causes. Finally, the well-established calcium chloride technique for the activation of washed suspensions of the spore-bearing anaërobes (Bullock and Cramer, 1919; Tenbroeck and Bauer, 1926; Fildes, 1927) was tried, and gave a thoroughly reliable method for inducing regular infection with *C. chauvœi*.

In attempts to produce spore growths for infection experiments, it was found, as reported in a previous paper, that some strains produce spores only with the greatest difficulty on any medium, while with others, up to 75 per cent. of spores can be obtained from surface growths on Dorset egg medium. Again, it has been shown in these experiments that spore production does not necessarily affect the powers of invasion. After repeated and rapid subculture over a period of several months strains originally producing 75 per cent. of spores may yield only 5 to 10 per cent., but the relative killing power of cultures with the low spore content remains apparently unaltered. It was further established that some strains when grown for long periods on artificial media become completely avirulent for guinea-pigs.

Of the various media used for growths for preliminary infection experiments, Dorset egg medium gave suspensions with the highest spore yield and the

greatest regularity in infecting powers. With 24- to 48-hour glucose-broth cultures, alteration in the bacterial cell is shown by the relatively rapid change from a Gram-positive to a Gram-negative phase.

PREPARATION OF SUSPENSIONS FOR INFECTION EXPERIMENTS.

Strains were grown anaërobically on chopped meat broth for 48 hours, subcultured on Dorset egg medium and incubated 48 hours under strict anaërobiosis. The liquid inoculum was carefully removed and discarded, and the surface growth removed by two washings with fresh normal saline. Gross particles were removed by light spinning and the suspensions then centrifuged, twice washed with saline, and resuspended in saline to an opacity five times greater than B.W. & Co.'s standard opacity tube No. 10. The suspensions were finally diluted with saline as required.

PREPARATION OF "O" ANTIGEN FOR VACCINATION.

Strains were grown on 1 per cent. glucose broth in quantities of approximately 1 litre incubated 14 to 16 hours under anaërobic conditions. A 10 per cent. inoculum of a 14- to 16-hour growth in glucose broth was used. Growths were centrifuged, twice washed in 200 c.c. distilled water, and resuspended in distilled water to opacity 10×10.

In preliminary vaccination experiments suspensions were treated for 30 minutes at 100° C. on the first day, and for 30 minutes at 90° C. on two successive days. For the final cross-immunity experiments, however, suspensions were steamed for 2 hours at 100° C. on one day. After heating, suspensions were adjusted to a salt concentration of 0·85 per cent. by the addition of 10 per cent. NaCl solution. Sterility tests were made on meat broth, incubated anaërobically for 3 days.

METHOD OF EXPERIMENT.

Guinea-pigs of 250 to 300 gm. were selected for vaccination. Three subcutaneous injections, of the undiluted suspension of opacity 10×10, were given in the left leg on alternate days, 1 c.c. at the first inoculation and 2 c.c. subsequently. Fourteen days later the animals received a test-dose intramuscularly on the opposite leg. The animals, as a rule, gained weight during the course of the immunization, and no ill-effects on their general health were noticeable. There were occasional swellings in the superficial inguinal region, which became markedly enlarged when the animals received the test dose. In some cases these swellings reached the size of a walnut, but disappeared within 14 days in the animals which survived the test dose.

For vaccinated animals the test suspension was used in a maximum opacity of 10×5, and also in dilutions of 1 in 10 and 1 in 100 of that opacity. The test dose was always given intramuscularly in a total volume of 1 c.c., and contained (1) 0·30 c.c. of the bacterial suspension in the required dilution, (2) 0·20 c.c. of a freshly prepared solution of 5 per cent. calcium chloride in distilled water, (3) 0·50 c.c. saline. Following the procedure of Robertson and Felix (1930)

in each experiment an indication of the infective power of the suspension was obtained by titrating the test dose used and higher dilutions of it in normal guinea-pigs. With increasing dilutions a point was reached where the suspension showed irregular powers of infection, and only those dilutions giving clear-cut results within 48 hours to 4 days after infection were taken as giving a reliable indication of the severity of the test.

RESULTS OF EXPERIMENTS WITH GUINEA-PIGS.

Table I records the results of experiments using vaccines prepared from one English bovine strain (G1) and one English ovine strain (M7). The suspensions used for vaccination were killed by steaming for 30 minutes at 100° C. on one day, and for 30 minutes at 90° C. on the two following days. The infecting suspension was from the English bovine strain G1. Six guinea-pigs from each batch of immunized animals received the undiluted fluid (opacity 10 × 5), while three guinea-pigs received a 1 in 10 dilution of this suspension. In the control guinea-pigs the infecting suspension was titrated up to the dilution of 1 in 10,000.

TABLE I.—*Vaccination Experiment with Pure "O" Antigen.*

Vaccine prepared from—	Number of guinea-pigs vaccinated.	Dilution of infecting suspension. (Strain G1 in opacity 10 × 5.)	Result.
Strain G1 (English bovine)	6	Undiluted	Four survived. One dead in 19 hrs., from typical *C. chauvœi* infection; one dead third day, not from *C. chauvœi* infection.
	3	1 in 10	All survived.
Strain M7 (English ovine)	6	Undiluted	,,
	3	1 in 10	,,
Controls in normal guinea-pigs.			
Titration of infecting suspension	2	Undiluted	Two dead in 18 hrs.
	2	1 in 10	Two dead in 24 hrs.
	2	1 in 100	Two dead in 24–36 hrs.
	2	1 in 1000	Two dead in 36–48 hrs.
	1	1 in 10,000	Survived.
Bacterial suspension without $CaCl_2$	1	0·30 c.c. of undiluted opacity 10 × 5	,,
$CaCl_2$ activator	1	..	,,

Test dose given intramuscularly in 1 c.c. total volume: 0·30 c.c., bacterial suspension; 0·20 c.c., 5 per cent. $CaCl_2$; 0·50 c.c., saline.

It is seen from the table that regular deaths occurred in the control guinea-pigs in from 18 to 48 hours with dilutions up to and including 1 in 1000. On the other hand, all the nine animals immunized with the heterologous strain (M7 ovine) survived, while of the nine guinea-pigs vaccinated with the homologous strain (G1 bovine), one died from typical *C. chauvœi* infection, and one from non-specific causes (the post-mortem examination on the third day showing no signs of a *C. chauvœi* infection).

Severe local reactions were not uncommon among vaccinated animals, involving in some cases a perforating gangrene which generally appeared within 24 hours, had contracted and healed within 48 hours, and from which the animals recovered completely. In the other vaccinated animals only a small local swelling appeared within 24 hours, which subsided by the 4th or 5th day. In striking contrast to these conditions was the frequent appearance of a perforating gangrene in the non-vaccinated controls, which in every case culminated in death.

In an analogous experiment using the same strains and vaccines for immunization, the infecting suspension was prepared from the English ovine strain M7. This suspension produced regular deaths among control guinea-pigs in dilutions up to 1 in 100. Only one typical *C. chauvœi* death occurred among the 16 vaccinated animals which were subjected to a test dose, ten and one hundred times stronger than the highest dilution producing regular deaths in the controls. There were five cases of perforating gangrene, all of which recovered, and with the remaining ten animals varying degrees of local swelling appeared, and had subsided by the 4th or 5th day after infection.

These results established the value of washed and steamed suspensions of *C. chauvœi* as powerful immunizing agents, and showed that bovine and ovine strains of English origin were capable of inducing complete cross-immunity. The identity of the " O " antigen in these strains previously established by *in vitro* analysis had thus been corroborated by the protection experiment.

Two further points were investigated : firstly, whether the cross-immunity could be obtained with representative strains of ovine and bovine origin from other countries ; secondly, as it had been shown in a previous paper that the agglutinogenic value of the " O " antigen was not reduced by steaming to 100° C. for 2 to 3 hours, it was considered of interest to examine the effect of such prolonged heating on the immunogenic value of the " O " antigen of *C. chauvœi* vaccines.

Table II records the results of an experiment with vaccines killed by steaming for 2 hours at 100° C. All vaccinated animals received a test dose of the English bovine strain G1 14 days after the last inoculation. The infecting capacity was such that the suspension of opacity 10 × 5 diluted 1 in 10,000 produced typical deaths in normal guinea-pigs in from 40 hours to 4 days, while all lower dilutions killed within 18 to 48 hours. Of 35 vaccinated animals 4 died from typical *C. chauvœi* infection in from 2 to 4 days ; in these cases severe local swellings persisted without the appearance of a perforating gangrene. Twelve other vaccinated guinea-pigs developed the typical perforating gangrene, from which they recovered completely. The remainder showed varying degrees of local reaction, which disappeared in from 4 to 7 days.

TABLE II.—*Vaccination Experiment Using "O" Antigens Steamed 2 Hours at 100° C.*

Vaccine prepared from—	Number of guinea-pigs vaccinated.	Dilution of infecting suspension. (Strain G1 in opacity 10 × 5.)	Result.
Strain G1 (English bovine	4	1 in 10	All survived.
	2	1 in 100	Both survived.
Strain A9 (S. African ovine)	5	1 in 10	Three survived. One dead second day, one dead third day. Typical *C. chauvœi* infections.
	3	1 in 100	All survived.
Strain A10 (S. African bovine	5	1 in 10	Four survived. One dead fourth day—typical *C. chauvœi* infection.
	3	1 in 100	Two survived. One dead second day—typical *C. chauvœi* infection.
Strain 3265 (Australian bovine)	5	1 in 10	All survived.
	3	1 in 100	,,
Strain 3266 (Australian ovine)	4	1 in 10	,,
	3	1 in 100	,,
Controls in normal guinea-pigs.			
Titration of infecting suspension	3	1 in 10	One dead in 24 hrs.; one dead in 40 hrs.; one dead in 48 hrs.
	3	1 in 100	One dead in 18 hrs.; one dead in 24 hrs.; one dead in 30 hrs.
	2	1 in 1000	Both dead within 40 hrs.
	2	1 in 10,000	One dead in 40 hrs.; one dead fourth day.
	2	1 in 100,000	Both survived.
Bacterial suspension without $CaCl_2$ activator	2	0·30 c.c. of undiluted opacity 10 × 5	,,
$CaCl_2$ activator	1	..	Survived.

Test dose given intramuscularly in 1 c.c. total volume: 0·30 c.c., bacterial suspension; 0·20 c.c., 5 per cent. $CaCl_2$; 0·50 c.c., saline.

EXPERIMENTS WITH MICE.

Attempts were made to immunize mice by similar methods. Vaccines were prepared from strains G1 and M7. For each strain two lots of 15 to 20 mice were used, one for subcutaneous and one for intravenous vaccination. Three inoculations were given on alternate days; subcutaneously the dose was 0·50 c.c. of vaccine of opacity 10 × 10, and intravenously 0·50 c.c. of opacity 10. Eleven days after the last inoculation the animals were tested with infecting suspensions of their respective homologous strains. In view of their high susceptibility to *C. chauvœi* infection a less severe test had to be applied, and dilutions of the bacillary suspensions were used from opacity 10 × 5 diluted 1 in 1000 to opacity 10 × 5 diluted 1 in 5,000,000. These experiments failed to show any protection. Further attempts were made by increasing the number and quantities of doses given by the intravenous route; again the results showed no protection.

DISCUSSION.

Since the introduction of the formolized whole culture vaccine of Leclainche and Vallée in 1925 there has been a better appreciation of the value of the bacillary body in blackleg prophylaxis. McEwen (1926), Green (1929) and Bosworth (1930) have indicated the immunizing value of suspensions of *C. chauvœi* freed from the so-called "aggressin." The view is still held, however, that in formolized vaccines there are two immunizing agents, viz. the bacillary body and the "aggressin."

In the case of the "aggressin" the claim is primarily based on the fact that *C. chauvœi* filtrates are effective immunizing agents. There is, however, no evidence to show that the immunizing action of these filtrates is due to some agent other than the autolysed bacillary antigen. It is clear that the filtrates obtained from 4- to 21-day growths of *C. chauvœi* which are used as "aggressins" contain large quantities of the products of autolysis, since after 48 hours' growth in liquid media, the bacillary bodies are already in a Gram-negative phase, and microscopic examination shows that autolysis of the cells has commenced.

Felix and Robertson (1928) have shown that bacteria-free filtrates (toxin) of *C. tetani* from cultures incubated for 8 days, steamed at 100° C. for 2 hours and injected into rabbits, produced sera with high titre "O" antibodies as estimated by agglutination and complement-fixation. It is suggested, therefore, that bacteria-free "aggressins" of *C. chauvœi* owe their immunizing property to the autolysed "O" antigen which they contain.

Viljoen and Scheuber (1926) immunized sheep with highly potent "aggressins" (prepared from cultures incubated for a period of 3 weeks), and found that the maximal dose they could withstand was thirty certain killing doses of virulent whole-culture. From the present work it is seen that guinea-pigs immunized with washed and steamed bacillary suspensions can withstand one hundred to one thousand certain killing doses of an activated bacillary suspension. It remains, therefore, to be established whether an equally high degree of protection can be induced by pure "O" antigen in cattle and sheep.

The relative importance of a specific soluble toxin in infection with *C. chauvœi* and of its counterpart (antitoxin) in immunity is not clearly established. The evidence published by earlier workers may be differently interpreted. From the present work it would appear that the supposed toxin, when tested under optimal conditions in 18- to 36-hour glucose-broth culture, has a very weak power of initiating infection in guinea-pigs, in conjunction with the bacterial cells. These latter, however, when washed and activated with calcium chloride prove highly virulent.

The fact that the immunogenic value of *C. chauvœi* vaccines is not appreciably lowered by steaming at 100° C. for 2 hours appears to be of considerable practical importance. One of the difficulties experienced with formolized suspensions of spore-bearing anaërobes is the survival of spores. The use of steamed suspensions, therefore, which would provide a reliable, sterile and innocuous vaccine might lead to improved technique in prophylaxis. With *C. chauvœi* such vaccines need not be polyvalent, *i. e.* contain antigens from various strains, since the " O " antigen appears to be identical in all strains tested.

SUMMARY.

1. The pure " O " antigen of *C. chauvœi* is a powerful immunizing agent.

2. This antigen is identical in strains of bovine and ovine origin and induces complete cross-immunity with all strains tested.

3. Heating for prolonged periods up to 2 hours at 100° C. does not lower appreciably the immunogenic value of the " O " antigen.

4. Steamed suspensions of *C. chauvœi*, being reliable, sterile and innocuous, may prove of value as prophylactic vaccines in the field.

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THE BLOOD GUANIDINE IN HYPERTENSION.

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A COLORIMETRIC reaction for the estimation of minute amounts of guanidine such as are believed to occur in blood was first suggested by Marston (1925). Marston's reagent was modified by Weber (1927), who substituted potassium ferricyanide for the ferrocyanide and hydrogen peroxide of the original solution, and greatly increased its sensitivity. The reagent (1 vol. 10 per cent. sodium hydroxide, 1 vol. 10 per cent. sodium nitroprusside, 1 vol. 10 per cent. potassium ferricyanide, 9 vols. water) gives a reddish-brown colour with creatine, and a very slight trace of colour with creatinine.

A method for the estimation of "guanidine" in blood was subsequently published by Major and Weber (1927). The blood is deproteinized by the Folin-Wu method, the filtrate rendered alkaline with sodium hydroxide, and the guanidine quantitatively adsorbed by adding 0·5 gm. of Merck's purified blood charcoal. The charcoal is filtered off and the guanidine extracted from it by treatment with acidified alcohol. The alcohol is evaporated off, the residue taken up in water, and the guanidine determined colorimetrically. The creatine content of the final solution must be determined, since creatine is also adsorbed by the charcoal. The creatine value obtained $\times$ 0·12 is deducted from the uncorrected guanidine figure, since creatine gives approximately one-tenth as intense a colour with the reagent as guanidine. Adding 0·2–0·3 mgm. of guanidine to 100 c.c. of blood, they obtained an average recovery of 75–85 per cent.

It remains uncertain whether the so-called guanidine present in blood is actually guanidine, and whether it is present as guanidine or as a substituted methyl compound. As far as we are aware, the only attempt at identification of guanidine in the blood is that of Paton and Sharpe (1926), who claim to have isolated appreciable amounts of methyl-guanidine from blood as a picrate, identified by its crystalline form and its melting-point. The substance was increased in amount after parathyroidectomy. For the sake of brevity we shall refer to the substance giving a colour reaction with Weber's reagent as guanidine.

Pfiffner and Myers (1930), using a slightly modified form of the method and larger amounts of added guanidine, recovered, on the average, 75 per cent. Creatinine may be ignored, since the colour given by it is only 1/400th of that produced by a corresponding amount of guanidine. They believe that guanidine, though completely adsorbed by the charcoal, is not entirely extracted by the acidified alcohol.

In view of the somewhat unsatisfactory recoveries with the charcoal adsorption method reported by these authors, with which our own small experience of the method agrees, we decided to employ the method of guanidine estimation described by Minot and Cutler (1928), with slight modifications.

METHOD.

Ten c.c. of oxalated blood in 70 c.c. water is incubated for 15 minutes at 37° C. with soy flour. The blood is then deproteinized by the Folin-Wu method, HCl being used instead of H_2SO_4, in order to avoid charring during evaporation. Fifty c.c. of the filtrate, with the addition of 1 c.c. saturated sodium carbonate, is evaporated to dryness on a water-bath under a fan. The residue is repeatedly extracted with about 40 c.c. in all of hot absolute alcohol, rubbing up with a glass rod, the washings filtered, and the alcohol evaporated, carefully avoiding charring. The residue is taken up in 8 c.c. of water, 5 c.c. being used for the colorimetric estimation of guanidine by Weber's method. The solution is filtered after adding the nitroprusside reagent, since a slight flocculent turbidity frequently develops, which interferes with colorimetric estimation. Two c.c. of the final solution is used for the estimation of pre-formed creatinine, and 1 c.c. for the creatine estimation after autoclaving, both estimations being made by the usual picric method. The creatinine and creatine estimations were corrected by means of standard curves constructed by estimating known solutions.

The guanidine reading was corrected by deducting the creatine × 0·12, Major's factor, which we can corroborate.

The method is laborious, and involves three colorimetric estimations with their accompanying inaccuracies, but appears to give good recoveries for guanidine added to blood:

Added guanidine	0·5	mgm.	per 100 c.c.,	recovered	0·51	mgm.
,,	1·0	,,	,,	,,	0·89	,,
,,	1·0	,,	,,	,,	0·92	,,

The urease was found to contain no interfering substance. In solutions containing guanidine, creatine and creatinine in varying amounts, the guanidine could be quantitatively recovered, after correction had been made for the creatine present. The substance estimated as guanidine appears to be evenly distributed between cells and plasma; in one estimation, for instance, the whole blood contained 0·272 mgm. per 100 c.c., the red cells 0·269 mgm., and the plasma 0·247 mgm.

In view of the close chemical relationship between arginine and guanidine, arginine was examined and found to give a colour reaction with the reagent very similar to that given by guanidine, though less intense. The effect of arginine in the diet on the colour reaction in the blood was therefore investigated, since it appeared that the presence of this amino-acid in the blood might affect the reaction, or that its ingestion might lead to an increase in the actual guanidine content of the blood.

An arginine-rich diet was prepared, consisting of 2½ parts of dried soft herring-roe, 2½ parts of pea-meal, and 1 part bran, and given to rabbits. The mixture was readily eaten to the amount of about 50 gm. *per diem*. The animals received in addition 2 oz. cabbage, and water *ad lib.*

In one rabbit the blood guanidine rose from zero on a cabbage diet to 0·188 mgm. per 100 c.c. after 7 days on the arginine-rich diet; fell to 0·062 mgm. after 19 days on cabbage, and was 0·053 mgm. after a further 6 days on the

special diet. In another animal the blood guanidine rose from 0·081 mgm. per 100 c.c. on cabbage to 0·106 mgm. after 6 days on the arginine diet. The richly nitrogenous arginine diet appears to have been well digested and absorbed, since the blood-urea rose by 50–100 per cent. on the special diet, falling again when the animals were replaced on cabbage. The relatively slight increase in the blood guanidine on a diet exceedingly rich in arginine, suggests that such variations in the intake of this amino-acid as are likely to occur in man on a mixed diet will not appreciably affect the guanidine of his blood.

NORMAL BLOOD GUANIDINE.

The amount of guanidine present in normal blood is given by Weber (1927) as 0·07–0·17 mgm. per 100 c.c.; by Pfiffner and Myers (1930) as less than 0·2 mgm. per 100 c.c.; and by Dodd, Minot and Casparis (1932) as 0·3–0·4 mgm. per 100 c.c. (in children); while Major and Weber (1927), in 35 healthy individuals, found an average figure of 0·11 mgm. per 100 c.c. with a range of 0·02 to 0·19.

TABLE I.—*The Blood Guanidine of Normal Subjects: Results expressed in mgm. per 100 c.c. blood.*

Sex.	Age.	Urea.	Creatinine.	Total guanidine.	Corrected guanidine.
F.	33	29	..	0·320	0·166
M.	33	31	1·59	0·172	0·036
M.	23	..	..	0·173	0·019
				0·181	0·041
M.	22	..	..	0·351	0·189
				0·348	0·211
M.	45	38	1·65	0·188	—0·237
M.	48	—	1·33	0·260	0·070
M.	22	31	1·53	0·188	0·064
M.	27	38	1·53	0·179	0·058
M.	21	..	1·49	0·404	0·074
M.	28	36	1·74	0·205	0·147
M.	34	37	1·61	0·302	0·284
M.	30	36	8·47*	0·338	0·183
F.	19	23	1·38	0·190	—0·235

* 3 gm. creatinine by mouth previously.

In 11 of 13 healthy individuals (Table I), bled at noon, we obtained an average figure of 0·117 mgm., with extremes of 0·036 to 0·284. In 9 of these the guanidine was less than 0·2 mgm. per 100 c.c.

In two cases a negative value for guanidine was obtained after correction for creatine. A similar negative value was also found in two of our pathological cases. Apart from these four instances, our guanidine figures have always been positive, and we believe that a degree of colour is produced in blood filtrates by Weber's reagent, which cannot be entirely accounted for by the creatine present. The negative values may be attributable to unavoidable inaccuracies in the method, or to the possible intervention of some chromogenic substance in the creatine estimation. In view of the exceedingly minute

amounts of guanidine to be estimated and the well-known inaccuracies of colorimetric methods, the actual figures given for guanidine must be, at best, approximate. The chief difficulty appears to lie in the creatine estimation.

THE BLOOD GUANIDINE IN HYPERTENSION.

Though studies of the blood guanidine have been made in tetany, in alimentary intoxication in infants, and in hepatic lesions, a special interest attaches to the possible *rôle* of this substance in hypertensive subjects.

There is a considerable body of evidence that guanidine is a pressor substance, though the mechanism of its action is disputed (Nakazawa and Abe, 1928). Major and Weber (1927), injecting methyl-guanidine-sulphate into dogs in doses of 10 mgm. per kg., obtained a rise of blood-pressure persisting for more than two hours. In one case an increase in the blood guanidine from 0·14 to 0·35 mgm. per 100 c.c. was accompanied by a rise in blood-pressure of 40 mm. of Hg.; in another a similar blood-pressure rise was seen, with an increase in guanidine from 0·18 to 0·47 mgm. Applying these findings to an investigation of hypertension in man, he found that of 35 hypertensive subjects, 18 showed a blood guanidine in excess of his highest normal figure. In one case a blood guanidine as high as 2·3 mgm. was found. In every instance in which the blood guanidine exceeded 1 mgm. gross nitrogenous retention was present, but a perusal of his figures shows that no constant relationship was present between the blood guanidine concentration and the blood non-protein nitrogen. In a subsequent paper Major (1929) gives the results of an investigation of the renal function in essential hypertension, and states that many hypertensive subjects, though showing no increase in blood-urea or creatinine, proved on testing to be unable to excrete creatinine injected intravenously as efficiently as normals, and also to show a poor excretion of methyl-guanidine given by mouth. He was unable to determine whether this impaired excretion was the cause of hypertension, or merely the result of a pre-existing hypertensive condition. In his latest paper (1932) he states that he has never seen a case of chronic nephritis with hypertension which failed to show an increase of blood guanidine, and that he has never seen an increased blood guanidine in a person with a normal blood-pressure. At the same time he fairly admits that it is not proved that the substance estimated is actually guanidine.

Major's results have so far received little corroboration. Pfiffner and Myers (1930) found a blood guanidine of 0·2 to 0·6 mgm. per 100 c.c. in 8 hypertensive subjects with the slightly increased average figure of 0·33 mgm.; Remond, Colombies and Vives (1928) found that the blood guanidine was usually raised in hypertension, but that exceptions occur. They found no relationship between the increase of guanidine and Ambard's coefficient, and felt justified in assuming that the increase is not due to defective renal function.

In Table II are summarized the results obtained in 17 hypertensive individuals. The first 12 cases we classified as essential hypertension, Cases 13 and 14 as hypertensive cardiac failure, and the remainder, on renal findings or retinal changes, as malignant hypertension. The average guanidine content of the blood in these patients was 0·233 mgm. per 100 c.c. (23 determinations), with extremes of 0·023 to 0·790. In 9 of the 23 determinations the guanidine

TABLE II.—*The Blood Guanidine of Hypertensive Subjects: Results expressed in mgm. per 100 c.c. Blood.*

No.	Sex.	Age.	Blood-pressure.	Urea.	Creatinine.	Total guanidine.	Corrected guanidine.
1	M.	55	196/100	35	1·55	0·466	0·360
			206/112	..	..	0·347	0·199
2	M.	57	180/96	28	1·59	0·186	0·111
3	F.	45	176/108	35	1·50	0·193	0·105
4	F.	59	222/122	25	1·43	0·163	0·077
5	F.	29	168/102	24	1·50	0·337	0·183
6	F.	60	204/106	42	2·37	0·696	0·376
7	M.	63	262/150	28	1·55	0·184	0·077
8	M.	57	226/124	45	2·40	0·505	0·388
9	F.	53	153/84	40	..	0·182	..
10	M.	40	176/94	39	1·60	0·320	0·312
11	M.	44	232/158	37	..	0·344	0·134
			218/154	42	1·77	0·186	0·102
			232/156	43	1·56	0·307	0·169
12	F.	58	216/124	46	2·34	0·578	0·331
13	M.	62	218/136	55	1·50	0·179	0·072
14	M.	33	202/154	29	..	0·180	0·023
15	M.	54	178/94	56	1·64	0·511	0·458
16	F.	48	300/160	22	2·48	0·552	0·301
17	M.	34	216/132	21	3·37	1·420	0·690
						1·440	0·790
			210/120	..	1·53	0·352	0·050
			206/116	..	1·77	0·333	0·173
			200/124	40	1·65	0·522	0·438

content exceeded the highest found in the normals. In 3 cases the estimation was repeated on one or more occasions after a few weeks' interval. In one case (11) very definite fluctuations occurred in the guanidine without corresponding changes in blood-pressure, and in another (17) the guanidine figure varied between the two extremes of the series, 0·05 to 0·79 mgm., without appreciable change in the hypertension.

TABLE III.—*The Blood Guanidine in Pre-eclampsia and Eclampsia: Results expressed in mgm. per 100 c.c.*

No.	Age.	Blood-pressure.	Urea.	Creatinine.	Total guanidine.	Corrected guanidine.
18	21	152/86	25	1·53	0·170	0·016
19	25	146/94	23	1·59	0·163	0·101
20	35	165/120	17	1·83	0·211	0·144
21	30	196/136	57	2·10	0·326	0·018
		208/132	30	1·70	0·172	0·172

THE BLOOD GUANIDINE IN ECLAMPSIA AND NEPHRITIS.

In 3 cases of pre-eclampsia and 1 case of eclampsia the guanidine content of the blood was low (Table III). In the eclamptic (21) the first sample of

blood was obtained within 12 hours of the fits, and showed a guanidine content of 0·018 mgm. only. It does not therefore seem possible to invoke the convulsant action of guanidine described by Major in explanation of the eclamptic convulsion.

TABLE IV.—*The Blood Guanidine in Nitrogenous Retention: Results expressed in mgm. per 100 c.c. Blood.*

No.	Sex.	Age.	Blood-pressure.	Urea.	Creatinine.	Total guanidine.	Corrected guanidine.
22	M.	31	216/162	86	3·84	0·188	—0·246
						0·184	—0·182
				176	6·42	0·640	0·430
23	M.	16	130/102	454	28·50	1·360	1·320
24	M.	57	112/64	93	3·26	0·191	—0·030
						0·191	—0·038
25	M.	55	140/90	220	6·17	0·533	0·107
26	M.	18	148/96	85	3·95	0·178	0·100
27	M.	23	190/118	102	3·30	0·356	0·072
28	M.	17	148/102	43	1·65	0·180	0·030

Lastly, a few cases of renal disease showing definite nitrogenous retention were examined since, if the guanidine increase in the blood of certain hypertensives is due to renal inadequacy, such cases should show a definitely raised blood guanidine content (Table IV). Of these cases, Nos. 22 and 23 were suffering from chronic glomerulo-nephritis, 24 and 25 from pyelonephritis, and the remainder from acute glomerulo-nephritis. Two only showed a rise of blood guanidine, both cases of chronic glomerulo-nephritis. In the second of these cases (23) the very high figure of 1·32 mgm. per 100 c.c. was obtained in association with gross nitrogenous retention. The blood-pressure was not raised, but the patient was moribund. In acute glomerulo-nephritis nitrogenous retention and hypertension were not associated with an increased guanidine in the few cases examined, and no rise of guanidine was seen in the nitrogenous retention of pyelonephritis. Though few in number, these observations do not suggest any direct relationship between nitrogenous retention and an increased blood guanidine.

In view of the lack of constancy of an increase of blood guanidine in the hypertensive subjects, and the absence of correlation between variations in the guanidine content and the height of the blood-pressure, it seems unlikely that the rise in blood-pressure is attributable to an excess of this body. The increased guanidine content seen in the blood of some hypertensive subjects is as yet unexplained, but is not apparently due to renal functional failure.

CONCLUSIONS.

1. The blood "guanidine," as estimated by the method of Minot and Cutler, was found to exceed the normal upper limit in 9 of 23 determinations on the blood of hypertensive subjects.

2. In successive determinations on individual hypertensive subjects, gross fluctuations in blood guanidine may occur without corresponding alterations in the blood-pressure.

3. The blood guanidine tends to be low in acute glomerulo-nephritis, pre-eclampsia and eclampsia.

4. In cases of nitrogenous retention the blood guanidine is not necessarily increased.

5. Arginine-rich food, given to rabbits, does not appreciably increase the guanidine content of their blood.

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A HEAT-STABLE PRECIPITATING SUBSTANCE EXTRACTED FROM VACCINIA VIRUS.

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THE disadvantages and dangers attendant upon vaccination with virulent calf-lymph have provoked many attempts to find some satisfactory method of producing immunity to smallpox without the use of living virus. Most of the attempts have been concerned with virus inactivated by heat or by chemical antiseptics, but the conflicting nature of the results recorded suggests that the immunity produced in some of the experiments may have been due to small quantities of living virus which had survived the inactivation process. Some workers, however, have claimed immunizing power for extracts of vaccine

lymph which almost certainly were devoid of living virus, since the process of extraction involved boiling (Torikata, 1917 ; Nakagawa, 1924–25).

During the course of experiments with a testicular strain of neurovaccine an extract was obtained which gave a sharply specific precipitation reaction when mixed with homologous immune serum. The active principle was found to withstand repeated boiling, and was considered to be similar to, if not identical with, the "Koktoprecipitinogen" obtained from calf-lymph by the authors cited above and by Tomarkin and Suárez (1917). An attempt was therefore made to immunize rabbits with this heat-stable precipitating substance, and experiments were made to gain further information on the immunity response of infected animals.

PREPARATION OF THE PRECIPITATING SUBSTANCE.

Rabbits are inoculated intratesticularly with testicular vaccinia virus, about 1 c.c. of a 1/100 emulsion in saline being injected into each testis. On the fourth day after infection the testes are removed, weighed and ground with 0·85 per cent. saline to make a 5 per cent. emulsion. The emulsion is transferred to a strong corked bottle, and 5 per cent. ether is added to prevent the growth of any accidental bacterial contaminants; it is then incubated at 37° C. for about 7 days, the bottle being shaken thoroughly each day. During this period of incubation considerable autolysis of the tissue occurs. The emulsion is then centrifuged, and the supernatant fluid, after being adjusted to pH 5·0 to 5·5 by the addition of HCl, is boiled for 3 minutes. The heavy coagulum which forms is centrifuged off, and the supernatant fluid, brought to a reaction of pH 8·0 by addition of NaOH, is boiled again. This usually results in a further slight precipitate, which is removed by centrifuging or by filtration through paper. The filtrate, now clear and colourless, is adjusted to neutrality, and distributed in tubes which are hermetically sealed. These are immersed in boiling water for 5 minutes to ensure sterility, and are then stored until required in the ice-chest.

This method has been found to yield precipitating extracts with fairly constant activity which keep indefinitely without detectable change. It is possible to obtain the precipitating substance by boiling fresh testicular emulsions without waiting for the autolysis which occurs during incubation, but such fresh extracts are more variable in their activity.

An attempt was made to increase the yield of precipitating substance by digesting the infected testis with trypsin at a reaction of about pH 8·4. The products obtained, however, possessed only about the same degree of activity as those prepared by autolysis.

THE PRECIPITATION REACTION.

Dilutions of extract and of serum are made in 0·85 per cent. saline, and 0·5 c.c. quantities of each reagent mixed in Dreyer's agglutination tubes. The mixtures are incubated in a water-bath at a temperature of 37° to 56° C.; between these limits the temperature has no appreciable influence on the speed and degree of the reaction. The first sign of actual precipitation occurs in

about 3 hours, and consists of a very fine granular precipitate visible only by means of a hand lens, though previous to this some of the mixtures become distinctly opalescent. The final reading is taken after incubation overnight when the precipitates have settled to the bottom of the tubes. By rotating each tube in turn the small floccules rise as a whorl, and are easily visible to the naked eye if viewed by transmitted light against a black background. Control mixtures of extract and saline, extract and normal rabbit serum and immune serum and saline are always included and should remain quite clear. It is important that all reagents be freed from visible particulate matter; this is done by filtering sera shortly before use through either a Seitz asbestos disc, or a "Gradocol" membrane of average pore size 0·45μ (Elford, 1931).

The following record of an actual experiment shows the range of the reaction when a good hyperimmune serum is used.

Experiment.—Hyperimmune serum R 1444 filtered through 0·45μ "Gradocol" membrane before use.

Temperature of water-bath 50° C.

Readings taken after 18 hours' incubation:

	Serum dilutions.							
	1/4.	1/2.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256
½	+++	+++	+++	+++	++	+	±	−
¼	++	++	++	++	+	±	−	−
1/8	+	++	+	+	±	?	−	−
1/16	±	∓	±	?	−	−	−	−
1/32	op.	−	−	−	−	−	−	−
1/64	sl. op.	−	−	−	−	−	−	−
1/128	tr. op.	−	−	−	−	−	−	−
1/256	−	−	−	−	−	−	−	−

Controls.

Extract and normal rabbit serum negative.
,, and saline negative.
Immune serum and saline negative.

+ signs indicate comparative amount of precipitate; op., sl. op., tr. op. indicate diminishing degrees of opalescence.

It will be seen that the highest dilution of extract to yield any precipitate was 1 in 16, whereas a comparable amount of precipitate was given by a 1 in 128 dilution of serum. For this reason routine tests have been limited to mixtures containing equal parts of undiluted extract and increasing dilutions of serum.

The specificity of the reaction has been repeatedly demonstrated by the failure of the extract to form a precipitate with any sera other than vaccinia immune sera. In addition extracts of normal rabbits' testes and rabbits' testes infected with herpes virus, made by the same technique as the vaccinial extracts, have consistently failed to precipitate even with the most potent vaccinial hyperimmune sera. On the other hand, extracts of herpes testis and of dog distemper ferret spleen show evidence of a slight reaction when

mixed with their homologous antisera, suggesting the presence of similar precipitating substances in tissues containing these viruses, but the reactions are so slight and irregular that further work with them has been suspended pending the evolution of a method of concentrating the active principle.

To obtain a good precipitating serum hyperimmunization is necessary. Sera obtained from rabbits which have recovered from vaccinial infection vary greatly as regards their precipitating potency; some of them indeed fail entirely to give a precipitation reaction, although possessing strong neutralizing power. Such a rabbit, however, if given a course of three or four intravenous inoculations of virus at weekly intervals, will yield a potent precipitating serum which is best harvested about a week after the last injection.

Physical and Chemical Properties of the Precipitating Substance.

Heat resistance.—An experiment was made to test the heat resistance of the active principle. An extract was distributed in 4-c.c. quantities into four ampoules, which were then sealed in the blow-lamp. Three ampoules were totally submerged in boiling water, and one was removed at the end of 5, 15 and 30 minutes respectively. The fourth ampoule was autoclaved at 15 to 20 lb. pressure for 15 minutes. Each sample was then tested against the same hyperimmune serum.

	Dilutions of serum.						Controls with normal serum.
	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	
Antigen boiled 5 min.	+	+	+	+	+	±	—
,, ,, 15 ,,	+	+	+	+	+	±	—
,, ,, 30 ,,	±	+	+	+	+	±	—
,, autoclaved	—	—	—	—	—	—	—

It appears from this experiment that the precipitating substance will withstand boiling for 30 minutes without any appreciable reduction of potency, but that its activity is totally destroyed by autoclaving for 15 minutes at 15 to 20 lb. pressure.

Filterability.—An attempt was made to gain some idea of the order of size of the unit of the precipitable substance by filtration experiments. Samples of extract, 5 c.c. in each case, were filtered through various "Gradocol" membranes of different pore size under a positive pressure of 2 atmospheres. The filtrates were then tested in parallel with an unfiltered control sample against a hyperimmune serum:

	Dilution of serum.							Controls.
	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	
Unfiltered antigen	+	+	+	+	+	±	—	—
0·25μ membrane filtrate	+	+	+	+	+	±	—	—
0·125μ ,, ,,	+	+	+	+	+	±	—	—
0·095μ ,, ,,	±	±	±	±	±	±	—	—
0·050μ ,, ,,	∓	—	—	—	—	—	—	—
0·009μ ,, ,,	op.	—	—	—	—	—	—	—

These results appeared to indicate that the units of precipitating substance have a size of approximately 0·04μ (Elford, 1932), but in view of the filtration results of Elford with different viruses, it was thought that the failure of the substance to pass the tighter membrane might have been dependent not so much upon the particle size, as upon absorption effects due to the nature of the continuous phase and the small quantities filtered. A second experiment was therefore made in which equal quantities of an extract and Hartley's broth were mixed together, and 10 c.c. quantities of the mixture were filtered through membranes of various average pore size:

	Dilutions of serum.								Control in normal rabbit serum.
	1/2.	1/4.	1/8.	1/16	1/32.	1/64.	1/128.	1/256.	
Unfiltered mixture .	++	++	++	++	+	+	±	−	−
0·050μ membrane filtrate.	++	++	++	++	+	+	C	−	−
0·018μ ,, ,,	++	++	++	+	+	±	−	−	−
0·009μ ,, ,,	+	+	±	∓	−	−	−	−	−
0·006μ ,, ,,	−	−	−	−	−	−	−	−	−
0·004μ ,, ,,	−	−	−	−	−	−	−	−	−

C = bacterial growth.

The results are interesting, in that they show clearly the great importance of extrinsic factors in the filterability of a disperse system as emphasized by Elford (1932). Under the conditions of the second filtration experiment the precipitating substance passed almost unchanged through a 0·018μ membrane and an appreciable amount passed through the 0·009μ membrane, whilst no trace could be detected in the 0·006μ membrane filtrate. Thus the active precipitating units are probably of the same order of size as molecules of egg albumin, which have an approximate size of 0·004μ, and are almost completely retained by a 0·006μ membrane. Moreover, the sharp filtration end-point indicates considerable uniformity of particle size of the disperse phase.

Tests for protein and carbohydrate radicles.—Extracts were tested repeatedly by the nitric acid ring test and the salicyl sulphonic acid test with entirely negative results. Various tests for protein and carbohydrate radicles were then made with the following results:

Biuret test, +.
Sakaguchi test for arginine, +
Millon test for tyrosine, +.
Adamkiewicz test for tryptophan—.
Sulphide reaction for cystine, −.
Molisch test for carbohydrate, +.

The extracts therefore contain both protein and carbohydrate radicles, and it is as yet unknown which is responsible for the precipitating activity.

ATTEMPTS TO IMMUNIZE RABBITS WITH THE PRECIPITATING EXTRACTS.

Experimental Details.

Animals.—Three normal rabbits bred at the Institute farm and therefore known to have had no contact with experimentally infected animals were

obtained. They were kept in an isolation room throughout the course of the experiment so as to avoid any possibility of contact infection with vaccinia virus.

Precipitating extract.—The same batch of extract was used for all the inoculations and serum tests. It was tested for the presence of any surviving virus by inoculating 1 c.c. into the right testis and 0·4 c.c. intradermally into the skin of the flank of a normal rabbit. No sign of reaction appeared in either testis or skin. On the 6th day after inoculation the animal was killed and the right testis examined histologically. The sections revealed no pathological change.

Inoculations and bleedings.—Each of the three rabbits was bled at the start of the experiment. In each case the serum was tested, and found to contain neither neutralizing power against vaccinia virus nor detectable precipitating antibodies when tested with the precipitating extract. These samples of normal serum were stored in the cold room for use in the subsequent comparative tests.

Rabbits Nos. 1 and 2 then received a course of intravenous inoculations of the precipitating extract, from 3 c.c. to 5 c.c. being given at approximately weekly intervals over a period of seven weeks. Rabbit No. 3 was given three similar intravenous inoculations, followed by four doses intraperitoneally. Seven days after the last inoculation each rabbit was bled, and the serum of each, after filtration through a Seitz filter, was tested for both precipitating and neutralizing antibodies as described below.

The animals were then rested for 3 months, during which period rabbit No. 2 died of an intercurrent infection. Rabbit No. 1 then received two further doses of 5 c.c. given intravenously, whilst rabbit No. 3 received doses of 5 c.c. and 7 c.c. intraperitoneally. They were bled 3 weeks after the second inoculation and their sera were examined as before.

Tests of sera for vaccinial antibodies.—The tests for precipitating antibodies were made by mixing undiluted extract with increasing dilutions of the sera.

In the tests for the presence of neutralizing antibodies, each of the sera obtained after the first course of immunizing inoculations was examined in parallel with the normal serum obtained from the same rabbit at the beginning of the experiment. Two series of serum virus mixtures were made, one containing various dilutions of a vaccinial testis emulsion with equal quantities of the test serum, the other containing the same dilutions of virus emulsion with equal quantities of the normal serum. The mixtures of one series were inoculated intradermally along one flank of a normal test rabbit, those of the other series into corresponding skin sites of the opposite flank. A direct comparison of the neutralizing effects of the test serum and the control serum could therefore be made on the same test rabbit, errors due to varying animal susceptibility being thus eliminated. By the same technique the sera obtained after the second course of immunizing inoculations were compared with sera from the first test bleedings.

Results.

Precipitating antibodies.—From none of the three rabbits was serum obtained which gave any sign of the precipitation reaction. In all the tests

a known positive serum was included, which always gave typical precipitation up to a dilution of 1 in 32 to 1 in 64.

Neutralizing antibodies.—The results of tests for neutralizing antibodies are shown in Table I.

TABLE I.—*Neutralizing Tests of Sera Obtained from Rabbits "Immunized" with Precipitating Extract.*

Experimental rabbit number.	Skin test rabbit number.	Serum.	Dilutions of virus in serum virus mixtures. 1/10⁴.	1/10⁵.	1/10⁶.	1/10⁷.
1	1420	Preliminary bleeding	+++	++	+	±
		1st test bleeding	+++	++	+	±
	1426	1st ,, ,,	+++	++	+	±
		2nd ,, ,,	+++	++	+	±
2	1421	Preliminary bleeding	+++	++	+	−
		1st test bleeding	+++	++	+	−
3	1422	Preliminary bleeding	+++	++	+	−
		1st test bleeding	+++	++	+	±
	1427	1st ,, ,,	+++	++	+	±
		2nd ,, ,,	++	+	±	−
	1432	1st ,, ,,	+++	++	+	±
		2nd ,, ,,	++	+	±	−
	1433	1st ,, ,,	+++	++	+	±
		2nd ,, ,,	++	+	+	−

Number of +.signs indicates comparative size of reaction.

With the sera from rabbits 1 and 2 no sign of any change following the attempted immunization could be detected. The serum of rabbit No. 3 showed slight irregularities of neutralizing power, but the changes during the period of hyperimmunization were not always in the same direction, so that they are not considered to have any immunological significance. The experiment therefore indicates that the precipitating extracts are devoid of antigenic power.

ARE THE PRECIPITATING AND PROTECTIVE ANTIBODIES IDENTICAL?

In order to obtain a good precipitating serum hyperimmunization is necessary; often the serum of a rabbit which has recovered from a severe generalized infection is totally devoid of precipitating power. In contrast to this are the facts that neutralizing antibodies in the serum of infected rabbits increase up to a maximum on about the seventh day following infection (Smith, 1929), and that subsequent hyperimmunization has little further effect upon the neutralizing power of the serum as revealed by the neutralization tests usually employed. It seemed possible that the phenomena of precipitation and virus neutralization might depend upon two separate antibodies in a hyperimmune serum. An attempt was made to test this point, for the existence of more than one antibody specific to vaccinia would necessitate the postulation of a complex antigenic structure of the virus itself.

The Effect of Hyperimmunization upon Serum Properties.

Four adult lop-eared rabbits of approximately equal weights were inoculated intravenously with 1 c.c. of a 1 in 20 saline emulsion of vaccinial testis. All the rabbits showed generalized skin-pocks on the fourth day after inoculation. Each was bled on the 25th day of the experiment and again on the 36th day. Precipitation tests were made on all the samples of serum; they showed that two of the rabbits had acquired fairly good precipitating sera, whilst the other two possessed sera which were very poor in precipitating power. The two latter animals were therefore hyperimmunized, whilst the former were kept without further treatment. Hyperimmunization consisted in seven doses of virus given intravenously over a period of seven weeks. Blood samples were obtained from all four rabbits after the second hyperimmunizing injection and again after the last dose. Thus each rabbit provided four different samples of serum, which were tested for both precipitating and neutralizing antibodies.

Tests for Precipitating Antibodies.

The results of tests for precipitating antibodies are given in Table II. They show that the two rabbits which yielded fairly good precipitating sera after the primary injection, and were therefore left without further antigenic stimulus, gradually lost their precipitating power, whereas the two rabbits with no detectable precipitating antibodies at the time of the first bleeding yielded good precipitating sera after a course of hyperimmunizing inoculations.

TABLE II.—*The Effect of Hyperimmunization upon the Precipitating Power of Rabbits' Serum.*

Rabbit number.	Treatment of rabbit.	Dilutions of serum tested for precipitation.																			
		1st bleeding (16.2.31).				2nd bleeding (27.2.31).				3rd bleeding (12.3.31).				4th bleeding (20.4.31).							
		1/2.	1/4.	1/8.	1/16.	1/2.	1/4.	1/8.	1/16.	1/2.	1/4.	1/8.	1 16.	1/2.	1/4.	1/8.	1/16.	1/32			
1358	Single injection	+	+	±	−	++	+	±	−	+	+	±	−	+	±	−	−	−			
1361	„ „	+	+	±	±	++	+	±	±	+	+	±	−	±	±	−	−	−			
1360	Hyperimmunization after 2nd bleeding	−	−	−	−	+	−	−	−	+	−	−	−	++	+	±	−	−			
1363	Ditto	−	−	−	−	+	±	−	−	+	±	−	−	++	+	+	±	±			

\+ signs indicate degree of precipitation.

Tests for Neutralizing Antibodies.

Only the sera from the first and last bleedings were tested, so that there were 8 samples in all to be compared. The most fruitful source of error in comparative neutralization tests is the varying susceptibility of rabbits to infection with vaccinia virus. To eliminate this source of error all the serum samples were tested against a given dose of virus on one and the same test rabbit, different rabbits being used for different doses of virus. In this way small differences of neutralizing power were revealed by differences in the sizes of the reactions, which could not have been detected if different sera had been examined on different test animals. The results given in Table III show that the rise and fall of the neutralizing power ran parallel with the rise and fall of the precipitating power, suggesting that the same antibody is concerned with both neutralization and precipitation.

TABLE III.—*The Effect of Hyperimmunization upon the Neutralizing Power of Rabbits' Sera.*

Rabbit number.	Treatment of rabbits.	Test of serum from 1st bleeding (16.2.31). Virus dilutions.				Test of serum from last bleeding (20.4.31). Virus dilutions.			
		1/20.	1/200.	1/2000.	1/20,000.	1/20.	1/200.	1/2000.	1/20,000.
1358	Single injection	+	+	−	−	++	++	−	−
1361	,, ,,	+	+	−	−	++	++	−	−
1360	Hyperimmunization	++	++	−	−	+	+	−	−
1363	Ditto	+	+	−	−	+	±	−	−

+ signs indicate comparative sizes of skin reactions.

Antibody Absorption Experiments.

Many experiments were made in which it was attempted to absorb out the precipitating antibodies of a hyperimmune serum with the extract. The neutralizing power of the absorbed serum was then compared with that of a sample of the same serum treated similarly, except that saline or normal testis extract replaced the absorbing dose of precipitating extract.

The results are not given in detail, because they were so irregular as to afford no convincing evidence either for or against the unity of precipitating and neutralizing antibody. The volume of precipitating extract necessary to absorb out all or even most of the precipitins of a serum was so great, that the consequent dilution of the serum rendered neutralization tests of doubtful value. The results taken all in all were thought to support the "unity of the antibodies" indicated by the hyperimmunization experiments.

SKIN SENSITIVITY TO THE PRECIPITATING EXTRACTS.

Rabbits.—A rabbit which had previously received three courses of hyperimmunizing inoculations at long intervals was inoculated intradermally with 0·2 c.c. of a precipitating extract. A control inoculation of 0·2 c.c. of herpes testicular extract was made in another skin site. The precipitating extract called forth a striking reaction within 24 hours of the inoculation, consisting of a raised œdematous bright red area about 4 cm. in diameter, which took several days to fade entirely. The herpes extract gave rise to no reaction whatever.

Since this result was obtained all available vaccinia rabbits have been tested for skin sensitivity from time to time. The intervals between primary infection and intradermal test have varied from one month to over twelve months, so that the animals may be assumed to have had very varying degrees of immunity. The results obtained have been most irregular. More than half the rabbits showed no sign of skin sensitivity, whilst the remainder gave reactions varying from a slight blush at the inoculation site lasting only a few hours to a raised œdematous red area, easily palpable, and lasting for a few days. Extracts of herpes testes have never been found to elicit such reactions in vaccinial rabbits. The factors governing the degree of skin sensitivity remain obscure, for no clear relation between skin sensitivity and degree or duration of immunity

could be traced; excepting that the very vigorous reactions occurred only in rabbits which had at some time been hyperimmunized. The negative group, however, included rabbits infected at the same time and in the same manner as members of the positive group.

The irregularity of the results suggested that the skin sensitivity might depend upon a precise concentration of circulating antibodies in the test animal. Two rabbits were therefore inoculated intravenously with a virus emulsion; in both cases a generalized infection resulted. Intradermal tests with precipitable extract were then made daily for 7 days following the primary infection, that is, during the period in which circulating antibodies are known to rise from zero to a level near the maximum. For each test a new skin site was used. Neither rabbit showed any sign of reaction. They were re-tested after an interval of 3 weeks' when both gave a fleeting doubtful reaction.

Humans.—It has been shown repeatedly that skin reactions may be elicited in previously vaccinated subjects by inoculation with heated lymph (Knoepfelmacher, 1907; Pirquet, 1911; Force, 1927). Andervont and Rosenau (1930) studied this question in detail and demonstrated the specificity of the allergic reaction; they state, however, that the virus itself, though killed, is responsible, and obtained completely negative results with lymph freed from the virus by filtration. It was therefore decided to test the effect of intradermal inoculations of the boiled precipitating extracts in human volunteers.

It was found that all except one of the previously vaccinated persons tested were definitely skin-sensitive to the extracts, and the severity of the reactions in the early cases which received from 0·1 c.c. to 0·2 c.c. of undiluted extract necessitated a reduction of the test dose. Thus it appears that humans are more sensitive in this respect than immunized rabbits.

The reaction consists of a pink flush appearing at the inoculation site in about 12 hours, which thereafter increases in size and intensity, to attain its maximum in 24 to 48 hours. It then consists of a central zone, bright red in colour, distinctly raised and slightly tender, the size being of course largely dependent upon the quantity inoculated. This zone is surrounded by a peripheral zone pink in colour, not usually raised, though with a large dose it may be slightly œdematous. There is usually itching during the period of development of the reaction. After 48 hours the reaction fades slowly, sometimes taking 5 or 6 days before all traces have vanished.

All cases have received a control inoculation, consisting of an equal amount of normal rabbit testis extract or herpetic rabbit testis extract. These have produced no sign of reaction in the majority of cases, but occasionally there has been a slight transient reaction, which has entirely faded by the time the vaccinial reaction has reached a maximum.

It is difficult to find volunteers who have not previously been vaccinated, and up to the present only two such have been available for tests. In both these cases the precipitable extract caused no reaction whatsoever.

The results are therefore suggestive, but very many more tests on both vaccinated and non-vaccinated subjects must be made before it will be possible to correlate skin sensitivity and degree or level of immunity. It is hoped to do this as occasion offers.

TABLE IV.—*Human Skin Reactions to the Precipitating Extracts.*

Case.	Age.	Vaccination history.		Amount of inoculum. (c.c.)	Reaction.
		Primary vaccinations.	Revaccinations.		
W. S—	35	1915, good take	1922, negative	0·2	+++
C. H. A—	36	Infancy, good take	1922, positive	0·1	+++
P. H—	51	„ „	1909, 1910, 1911, 1912, 1915, all positive, ? allergic; 1919 ?	0·1	+++
W. F—	37	„ „	1914, negative	0·1	++
J. G—	32	„ „	1918, positive; 1929, negative	0·02	++
H. B—	29	„ „	1918, ±; 1930, negative	0·02	++
H—	28	1918 „	1924, ±	0·02	+
P. W—	40	Infancy „	1924, ±; 1929, negative	0·02	++
S—	27	„ „	1920, positive	0·02	++
B—	...	1912, ± take	1931, positive	0·02	—
F—	40	Infancy, good take	..	0·02	++
B—	7	1931, „	..	0·02	+++
W. J. E—	32	Infancy „	1918, negative	0·02	++
R—	21	*Nil*	..	0·02	—
P—	7	„	..	0·02	—

In last column + signs indicate size in mm. of central zone of reaction: + 5 to 10 mm.; ++ 10–20 mm.; +++ 20–30 mm.

DISCUSSION.

The present work largely confirms that of the previous workers, who have reported that a heat-stable precipitating substance can be extracted from tissues infected with vaccinia. Attempts to produce vaccinial immunity, however, by means of boiled precipitating extracts failed to support the claim that such "thermoprecipitin" is truly antigenic, and whilst admittedly the scope of the experiment was inadequate for obtaining definite proof, it was sufficient to show that the immunizing power of boiled "precipitinogen" is negligible.

Craigie (1932) has shown that Seitz filtrates of vaccinial material, although non-infective, contain a precipitating substance which reacts specifically with vaccinia immune sera. By cross-absorption experiments he has demonstrated that the antibodies concerned in this reaction are identical with the antibodies which cause agglutination of the washed virus bodies. Although Craigie reports that his precipitating antigen is thermolabile—"under certain circumstances at 56° C."—it would seem probable that it is essentially the same as the heat-stable active principle in the extracts described here. Craigie did not attempt to show the identity or dissimilarity of the agglutinating and precipitating antibody with the virus-neutralizing or protective antibody. Although the absorption experiments failed to yield definite proof on this

point, the experiments showing a parallel rise and fall of precipitating and neutralizing power of the sera of rabbits undergoing hyperimmunization suggest that one and the same antibody is responsible for all the immunity phenomena so far described. Moreover, they afford evidence that the precipitating substance is a part or a product of the virus bodies themselves. Were it not for the filtration results, it would be reasonable to assume that the substance consists of fragments of virus bodies broken up during the necrotic development of the vaccinial lesion, and during the autolysis of tissue in the first stage of extraction, but this is rendered most improbable by the great uniformity and extremely small size of the active principle. In this connection it is of interest that similar extracts prepared from dermovaccinial material apparently react to a much higher titre than do the neurovaccinial extracts described here (Tomarkin and Suárez, 1917), and that the heat-labile precipitating extracts of Craigie and Tulloch (1931) were very much more potent than any preparations obtained by the author from testicular virus either with or without heat extraction. Neurovaccine, however, is fully as infective for the rabbit as dermovaccine, usually more so, and hence may be assumed to be quite as rich in virus. These facts are entirely explicable on the hypothesis that the precipitable substance is a produce of the virus, varying quantitatively and possibly qualitatively with different virus strains. In many respects indeed the substance behaves like the bacterial haptenes, and it may be that it is of similar nature.

The results of the intradermal inoculations in human subjects offer a hope that extracts of value for diagnostic purposes might be obtained from the pathological tissues of other virus diseases.

SUMMARY.

1. Extracts of testicular vaccinia virus have been obtained which give a specific precipitation reaction with immune sera. The precipitating substance withstands repeated boiling.

2. Chemical tests show that both protein and carbohydrate radicles are present in the precipitating extracts.

3. Filtration experiments indicate that the precipitating substance consists of particles with an approximate size of 0·004μ.

4. The heat-stable extracts possess no antigenic power.

5. The study of serum changes during hyperimmunization affords evidence that precipitating antibodies are probably identical with virus-neutralizing antibodies.

6. Allergic skin reactions are elicited by inoculation of the extracts into previously vaccinated human subjects, but not in those who have never been vaccinated.

I wish to express my indebtedness to my colleague Dr. W. J. Elford, who carried out the various filtrations. Also to Dr. Kersley for his help in carrying out skin tests on human volunteers.

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THE SIZES OF DIFFERENT BACTERIOPHAGES.

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THERE are many references in the literature to work on the size of " the bacteriophage " : it would be as reasonable to talk of the size of " a bacterium," for there can be very few workers to-day who would agree with d'Herelle that all races of bacteriophage are representatives of a single species, the " *Bacteriophagum intestinale.*" Some writers on the subject have not even recorded what bacteria were attacked by the particular bacteriophage whose size they were estimating. Very few have considered the possibility that different bacteriophages might be of differing sizes. Biemond (1924), however, found evidence that one Shiga-phage studied by him was larger than another one ; and Burnet and McKie (1930, *b*) thought that their anomalous Salmonella phage S 13 was smaller than most others.

The earlier workers compared phages with other substances as regards their filterability through collodion membranes ; most of them reached untrustworthy conclusions through neglect of the fact that the presence of phage can be detected in far lower concentrations than is possible with the substances with which they were comparing it. It has thus been concluded that " the phage " is smaller than albumin (Bürgers and Bachmann, 1924), than strychnine nitrate (Stassano and de Beaufort, 1925), than hæmoglobin

and serum-albumin (Eliava and Suarez, 1927), than nucleo-protein and albumin (Jermoljewa, Bujanowskaja and Severin, 1932).

The majority of workers have, however, agreed more or less with d'Herelle's estimate of 20–30$\mu\mu$ as the diameter of "the bacteriophage." This conclusion has been reached mainly on the basis of filtration experiments through acetic collodion membranes; such membranes are, however, far from ideal for the purpose, as discussed by one of us elsewhere (Elford, 1931). Optical methods have been used by Bechhold and Villa (1925–6), who consider that "the phage" has a diameter of over 35$\mu\mu$. Hetler and Bronfenbrenner (1931) estimated the size of a coli-phage by a diffusion method. They believe that phage in broth filtrates is adsorbed to particles of bacterial protein of varying size, but that the ultimate units of purified phage may be as small as 1·2$\mu\mu$ in diameter. Reasons for doubting the validity of their conclusions will appear in the subsequent discussion.

EXPERIMENTAL METHODS.

The material studied has consisted of filtrates of lysed broth cultures of organisms. Experiments with "purified" phage will also be described. Most of the phages are amongst those studied by Dr. F. M. Burnet (1930, *a*); we are much indebted to him and to Prof. Topley for strains of these and other phages; also to Dr. A. P. Krueger for a strain of staphylococcal phage. Care was taken by repeated plating out and picking of single plaques to ensure that all the phages studied were really pure. Titres of phage were estimated by means of plaque-counts on agar plates; despite its defects, this seemed to us the best method available.

We confined our attention to phages which could be obtained in high titre —at least 10^7 particles per c.c.—and we were careful to ensure in the course of our work that they were maintained at their maximum titre and filtered at a pH of 7·4 to 7·6. The effect of variations in titre on the filterability of a

TABLE I.—*Phages Studied.*

Designation.	Organisms attacked.	Average plaque-size on 2% agar in mm.*	Titre ordinarily obtained particles/c.c.
Staph. K	*Staph. aureus*	0·1	10^9
C 13	Various dysentery bacilli, *B. coli* and Salmonellas, *cf.* Burnet, 1930, *a*	2·3	10^8
C 36		1·7	10^{10}
D 4		0·3	5×10^7
D 12		0·0†	10^9
D 13		1·5	10^8
D 20		1·7	10^7
D 48		1·1	10^9
D 54		0·35	10^{10}
S 13		3·9	5×10^7
S 41		0·2	10^9

* All but the first and last measured on plates sown with rough Flexner W.

† See remarks on p. 454.

virus had been forcibly brought home to us during earlier work with vaccinia (Elford and Andrewes, 1932); we took care, therefore, that our results with phages were not vitiated by fallacies arising in this quarter.

The phages shown in Table I were studied.

The several phages have been filtered through graded collodion membranes of the new type described by Elford (1931). (For convenience these membranes are referred to as gradocol membranes.) The form of ultrafilter employed was the same as in previous experiments (see Barnard and Elford, 1931). The membrane was supported on a perforated plate, and filtration conducted under positive nitrogen pressure of 76 cm. mercury. The experimental conditions were carefully controlled: the temperature was 18–20° C., the area of membrane surface (*i. e.* total area of holes in plate supporting the membrane) was 1·05 sq. cm., while the membrane thickness ranged from 0·12–0·15 mm. The analytical procedure consisted in collecting measured samples of filtrate, which were then tested quantitatively for the presence of phage by the method already indicated.

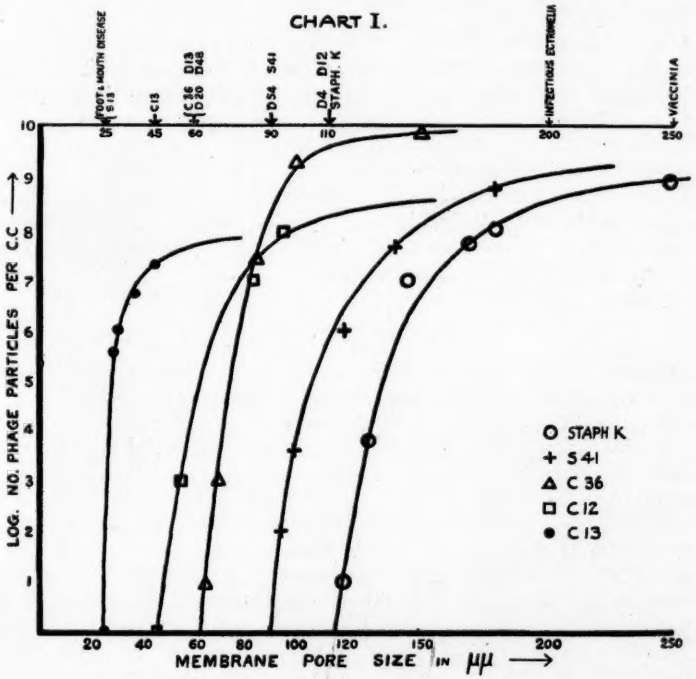
EXPERIMENTAL RESULTS.

The behaviour of the phages when filtered through membranes of graded porosities was found to be closely analogous to that exhibited by colloids (Elford, 1932). Thus in each experiment there was an initial phase during which the adsorbing capacity of the membrane became saturated, followed by the second phase of filtration through the equilibrated membrane. Using the standard conditions mentioned above and filtering always 5 c.c., we found that the phage passed membranes of progressively smaller porosities with no appreciable diminution in titre until a fairly definite point was reached, when a significant drop in concentration occurred. This invariably corresponded to a membrane porosity approximately twice that which ultimately proved to be the true "end-point." From this point onwards the filtration was analysed in detail in order to establish the absolute "end-point" in membrane permeability for the particular phage. The volume of filtrate was no longer restricted to 5 c.c., but much larger volumes were collected (25–30 c.c.). Analysis of successive samples of filtrate (1 or 2 c.c.) were made, and in this way it was possible to decide to what extent adsorption and true filtration respectively were responsible for the reduction in phage concentration. If this reduction is primarily due to adsorption, then the filtrate eventually attains to a phage titre comparable with the original, but when the true end-point is approached and true filtration predominates, the maximum phage concentration fails to reach the original value. Finally beyond a limiting permeability, the "end-point," the initial phage concentration and the volume filtered no longer affect the result, the filtrate being consistently inactive. The "end-points" in membrane permeability have been determined for each of the strains of phage described, and the data are charted collectively in Chart I, the logarithm of the maximum concentration of phage (expressed as number of particles per c.c.) in the filtrates being plotted against the pore sizes of membranes used. The curve for *Staph. k.* phage is representative of the group of phages *Staph. k.*, D 4 and D 12, whose filterabilities were not

sufficiently distinct to warrant their curves being separately plotted. Similarly the S 41 curve is representative of D 54 and S 41, and C 36 of the group C 36, D 13, D 20 and D 48. The well-defined differentiation of these curves, established in all cases for the most favourable filtration conditions procurable, indicates fundamental differences in the sizes of bacteriophages.

Uniformity of Size of Phage Particles.

The forms of the filtration curves (Chart I) and their clean separation suggest that the phage particles in any particular preparation are of a relatively uniform



and characteristic order of size. For instance, in the case of S 13 phage, a 25$\mu\mu$ membrane completely retained the phage, a 30$\mu\mu$ membrane permitted 1 per cent. to pass, a 35$\mu\mu$ membrane 50–75 per cent., while grades 45$\mu\mu$ and upwards yielded filtrates equivalent in titre to the original. These facts, particularly when one remembers that the membranes are not ideal filters with pores all exactly of the same dimension, provide strong evidence that the phage particles are very uniform in size. Bronfenbrenner (1927) considers that the active principle in bacteriophage preparations is not in its elementary form, but is attached to carrier particles. We think it very probable that some

phage may be adsorbed to bacterial fragments, in view of the observed fact that dead bacteria can adsorb phage irreversibly (Krueger, 1931). However, were all the phage attached to bacterial or broth proteins of varying sizes, then a gradual loss of activity would be expected as progressively less permeable membranes were employed. Further, all the strains of phage, particularly those developed in the same organism, might be expected to have the same apparent size. The results obtained in our studies are in contradiction to both these implications, different phages being retained by membranes of widely differing permeabilities.

Effect of Growth on Different Organisms.

Most of the phages we studied would attack more than one kind of organism. We accordingly performed filtration experiments to see whether the size of a given phage was or was not affected by varying the organism on which it was grown. No evidence pointing to such variation was obtained. S 13 grown on *B. sanguinarium* was retained by a 25$\mu\mu$ but not by a 30$\mu\mu$ membrane, just as when it was obtained from cultures of *B. dysenteriæ Flexner*. C 36, whether multiplying on *B. coli* or on dysentery bacilli, passed a 95$\mu\mu$, but not a 60$\mu\mu$ membrane. Similarly, no evidence of change of size was found in experiments with D 20 (grown on *B. coli* and *B. dysenteriæ Flexner*), nor with S 41 (grown on *B. ærtrycke* and *B. coli*).

Surface Adsorption.

The adsorption zones in the filtration curves of the various phages were all very comparable, and did not suggest that any difference in relative adsorbability could explain the wide differences in the end-points. Separate adsorption experiments in which broth suspensions of the phages in equivalent titre within the range pH 6–8 were shaken with known amounts of collodion particles (prepared according to Loeb, 1922) furnished supplementary evidence. No significant differences were found in the adsorption of the various phages at pH 7·6. Similar experiments with purified phages suspended in aqueous buffer solution showed that under these conditions the amount adsorbed was very considerably increased. Further reference will be made to this in explaining the behaviour of purified phages during filtration.

We concluded that the apparent differences in size amongst the several phages could not be ascribed to differences in their adsorbabilities.

Probable Sizes of Phages.

Their filtration curves having been determined and analysed under strictly comparable conditions, the phages were arranged in order of their relative sizes. Thus—

$$\left.\begin{matrix}\textit{Staph. k.}\\ \text{D 4}\\ \text{D 12}\end{matrix}\right\} > \left\{\begin{matrix}\text{D 54}\\ \text{S 41}\end{matrix}\right\} > \left\{\begin{matrix}\text{C 36}\\ \text{D 13}\\ \text{D 20}\\ \text{D 48}\end{matrix}\right\} > \text{C 13} > \text{S 13}.$$

The difficulties in assigning values for actual sizes from filtration data have been fully discussed by one of us elsewhere (Elford, 1932). The probable sizes of the phages (see Table II) have been deduced from the end-point determinations on the basis presented in that paper.

TABLE II.—*Probable Sizes of Phages.*

Phage.	End-point.	Probable size.
Staph. K, D 4, D 12	110μμ	50–75μμ
D 54, S 41	90μμ	30–45μμ
C 36, D 13, D 20, D 48	60μμ	20–30μμ
C 13	45μμ	15–20μμ
S 13	25μμ	8–12μμ

Resolution of Mixtures.

The sharply defined separation between the end-points made it possible in several instances to filter out one of the components from a mixture of phages by means of a membrane intermediate in grade between the end-points of the two constituents of the mixture. Table III gives some of the combinations which have been successfully resolved in this way.

TABLE III.—*Resolution of Mixtures.*

Mixture.	Membrane.	Test on filtrate.
Staph. K + S. 41	110μμ	S. 41 positive, Staph. K negative.
S. 41 + C 36	90μμ	C. 36 positive, S. 41 negative.
C. 36 + S. 13	45μμ	S. 13 positive, C. 36 negative.
D 13 + C 36	..	Could not be separated—filtered with equal facility.

Diffusion Experiments.

In order to check the relationships in size of phages indicated by the filtration experiments, measurements were made of the rates of diffusion of three representative phages (S 13, C 36 and *Staph. k.*). These phages were contained in the same broth medium, the C 36 and *Staph. k.* phages being diluted so that all these were present in equivalent concentration as indicated by the plaque count. They were allowed to diffuse into broth through a Jena sintered glass disc, a cell of the type described by McBain and Liu (1931) being used. Diffusion was allowed to proceed for 6 hours at 22° C., and after this period the amounts of each phage that had diffused were estimated. Four experiments were conducted, and the same size relationship was indicated as in the filtration experiments. Thus—

Rate of diffusion S 13 > C 36 > *Staph. k.*

Size relationship S 13 < C 36 < *Staph. k.*

The experiments were designed to furnish qualitative evidence only as to the relative sizes of the three phages, the quantitative investigation necessary for the calculation of their actual sizes being deferred.

EXPERIMENTS WITH PURIFIED PHAGES.

(a) *Purification by Electrodialysis.*

The method employed for the purification of phages by electrodialysis was that described by Krueger and Tamada (1929, *a*); their technique was carefully followed. These workers claimed that their agar extracts of purified phage showed increased activity, the titre being as much as 5×10^6 that of the original; in our experiments, on the contrary, no enhancement of activity was obtained. The titre of the purified extract was invariably of the order of one-hundredth that of the original. Typical protocols are given in Table IV.

TABLE IV.—*Purification by Electrodialysis into Agar.*

Experiment.	Initial titre of phage (C36).	Titre of purified phage.	Extracting medium.
1	10^8/c.c.	10^6/c.c.	Saline
2	10^8/c.c.	2×10^5/c.c.	,,
3	4×10^9/c.c.	10^6/c.c.	,,
4	5×10^9/c.c.	10^7/c.c.	Broth.

(b) *Purification by Washing.*

A method which has been found of much service in purifying viruses (Barnard and Elford, 1931) may be used also for the bacteriophages. It consists in selecting a membrane which will effectively retain the particular phage, and then washing away the protein material through the membrane. The phage retained above the membrane is repeatedly diluted in the chosen medium—which may be broth, saline, Ringer or water—the alternate process of diluting and filtering being continued until the concentration of protein is reduced below the minimum amount detectable by the Millon test. The titre of the residual washed phage made up to its original volume is seldom found to be less than 1/10 the initial value. If the washing is carried out in broth to remove the bacterial proteins the titre scarcely falls at all. The stabilities of purified phages vary according to the medium used for working. Broth stabilizes well, but saline, Ringer and water have varying effects. S 13, for instance, becomes inactivated very quickly in saline but persists well in broth. C 36, too, is much more stable in broth than in saline or Ringer.

Filtration of Purified Phages.

Purified preparations of C 36, S 13, *Staph. k.*, D 4, D 20 phages were filtered through graded membranes under parallel conditions with phages not purified so that the results were strictly comparable. The purified phages, whether

washed or electrodialysed, were found to filter precisely as did the untreated preparations. Membranes which effectively retained the latter in no instance permitted purified phage to pass, while the broth suspensions of purified phage filtered through more permeable membranes in a manner perfectly analogous with the straight phage filtrates. When suspended in water or saline, however, purified phage filtered less readily. The curves of filtration suggested that probably two factors were contributing to this result—increased adsorption and aggregation. The increased adsorption of phage by collodion from aqueous buffer solutions was demonstrated by the direct adsorption experiments which have already been mentioned.

Our results are in contradiction to those of Krueger and Tamada (1929, *b*), who found electrodialysed coli-phage to be more readily filterable through 4·5 and 5 per cent. acetic collodion membranes than untreated phage. Further, no evidence has been obtained of the dissociation of phage such as Hetler and Bronfenbrenner claim to have demonstrated in the case of coli-phage (1931). In so far as filtration is a criterion, purified phages appear not to be altered perceptibly in size from their natural condition.

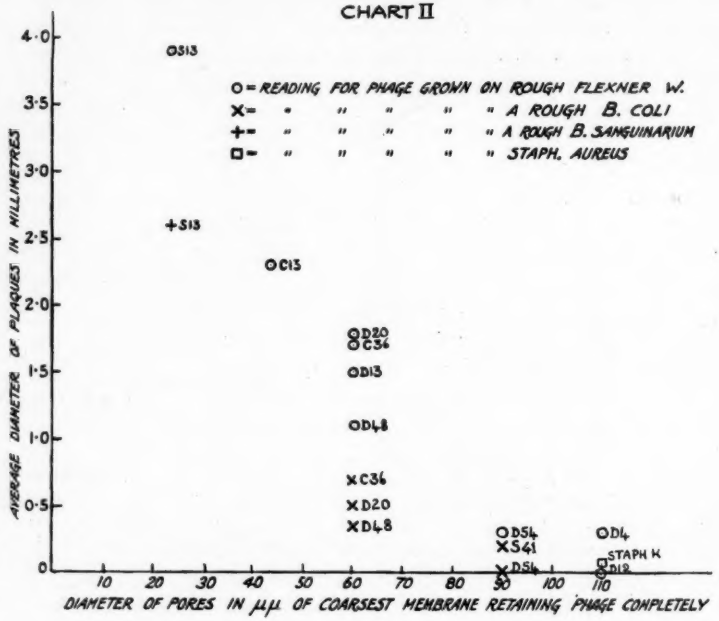
PHAGE-SIZE AND PLAQUE-SIZE.

Early in our work we were struck by the facts that a phage, S 13, which seemed by filtration experiments to be very small, produced very large plaques on agar, while our staphylococcal phage, the largest we then had, gave rise to minute plaques, and our coli-phage (C 36), which was apparently between them in size, produced plaques of intermediate size. A possible relationship between size of phage and size of plaque seemed accordingly to be worth some inquiry.

Measurement of Plaque-sizes.

Many factors determine the size of plaque which any given phage will produce on agar. When several phages are being compared it is necessary to standardize the conditions of the measurements in order to obtain comparable results. The technique we used was as follows: exactly 15 c.c. of 2 per cent. agar were poured into Petri dishes 8 cm. in diameter; plates were dried in the incubator, spread with organisms, and at the same time with a suitable dilution of phage; they were then again dried off. Readings were taken after 20 hours at 37° C. For the smaller plaques measurements were taken by the aid of a low power of a microscope with a micrometer eye-piece. Twenty plaques were measured and an average taken of the values obtained. As a general rule the heaviness of the seeding of bacteria and the time of taking the measurements were not factors greatly affecting the results; but the depth of agar in the plates and the concentration of the agar were most important. In most instances we also made measurements on plates poured with 20 c.c. of 1 per cent. agar, and the plaques obtained were very considerably larger than on the 15 c.c. 2 per cent. agar plates. Measurement of the smaller plaques was thus made easier, but it was found very hard to obtain reproducible results with the phages producing large plaques; probably we did not control sufficiently the drying of the plates.

With this exception the phages under study produced plaques of which the *average* diameter varied very little, though of course there was considerable variation around the mean on every plate. If, however, a phage was active against two different organisms, it commonly gave rise to different-sized plaques according to the organism attacked. Thus, a number of phages studied by us attacked a Flexner dysentery bacillus (rough W) as well as a rough *B. coli*. Almost all these phages produced larger plaques when acting on the dysentery than when attacking the *B. coli*. As mentioned earlier, the diameter of a phage particle is apparently irrespective of the organism attacked. In Chart



II one phage (D 12) is recorded as giving 0-sized plaques—plaques too small to measure. Such phages when tested undiluted could inhibit growth on a plate, but yet they could not produce discrete plaques when diluted and tested on 2 per cent.—or even 1 per cent.—agar. On still more dilute agar, however, (0·67 per cent.) one such phage could produce *tiny* plaques.

Relation of Phage-size to Plaque-size.

The relations we found to exist between phage-size and plaque-size are shown in Chart II. Four phages all producing plaques of intermediate size were retained by membranes with pores 60μμ in diameter. Two phages (C 13 and S 13) giving rise to larger plaques appeared from the filtration

experiments to be distinctly smaller, while our five largest phages all produced very small plaques. We are anxious not to draw too sweeping conclusions from these results. A relationship between plaque-size and phage-size certainly exists, and is particularly striking when one considers phages acting on a single organism (compare all the circles in Chart II); but we deemed it unwise to attempt to draw curves on the graph to illustrate the relationship, since the diameter of a phage-particle is certainly not the only factor affecting plaque-size under our standardized conditions. A small phage can probably travel by diffusion, and perhaps other means also, more rapidly than can larger ones, and thus tend to produce a large plaque ; but the rate of its multiplication and other factors will certainly come into play as well. It may be mentioned that when tested on Flexner WR on 2 per cent. agar, phage C 13, a definitely larger phage than S 13, regularly produced smaller plaques than did S 13 (see Chart II) ; but on 1 per cent. agar its plaques were larger on the average than were S 13's.

DISCUSSION.

The bacteriophages studied by us appear to be of the same order of size as the viruses attacking animals. For purposes of comparison the filtration end-points of three of these viruses are charted at the top of Chart I. But while most of the animal viruses so far measured seem to be greater than 75$\mu\mu$ in diameter, all our phages are probably smaller than this. It is of interest, however, that the smallest phage we have encountered (S 13) seems to be of almost exactly the same size as the smallest known virus, that of foot-and-mouth disease (*cf.* Galloway and Elford, 1931). While many animal viruses are now proving visible by more refined optical methods, our largest phages have apparently a diameter which does not bring them within the limits of optical resolution at present available.

Our observation that small phages tend to produce large plaques on agar, and conversely, may be of help to workers on the physical properties of phages, who may wish for any reason to study a very large or a very small phage.

SUMMARY.

1. The sizes of a number of bacteriophages have been estimated by the help of graded collodion membranes. Different phages appear to differ from one another in size, but the particles of any one phage seem to be very uniform in size, and not to vary even when the phage is grown on different organisms. The estimated diameter of the smallest phage we have studied (S 13) is 8–12$\mu\mu$, that of the largest is probably between 50 and 75$\mu\mu$.

2. Our conclusions as to the relative sizes of three phages have been confirmed by diffusion experiments.

3. Phages " purified " by two different methods show no evidence of change in size.

4. Size of a phage is an important factor in controlling the size of plaque produced. Small phages tend to give rise to large plaques on agar, and conversely.

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THE ISOLATION OF ORGANISMS IN THE PRESENCE OF *B. PROTEUS*.

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VARIOUS methods have in the past been recommended for preventing the swarming of *B. proteus* on the surface of solid media. A large number of such methods has recently been investigated by Krämer and Koch (1931), using 100 different strains of *B. proteus*. Their results showed that none of the methods was ideal, many of them only inhibiting swarming in a small proportion of the strains used, while those which inhibited swarming in all or nearly all strains also either inhibited the growth of other organisms, or so altered their colony characteristics as to make them difficult to recognize.

These authors described a method of their own, which depended on the addition of chloral hydrate to the medium. Using chloral hydrate blood-agar, they found that 76 per cent. of strains of *B. proteus* were prevented from swarming. This method has been tested again recently in this laboratory by Dr. Elizabeth Cooper, who found that, using concentrations of 1 in 600 to

1 in 1000 chloral hydrate in blood-agar, swarming was inhibited in only two out of twelve strains.

The method to be described was devised to overcome the disadvantages of these other media. It is applicable for the isolation of any organisms which are not obligate aërobes, and the deep colonies of which can be easily identified. With these limitations it should always be successful.

TECHNIQUE.

The method depends on the fact that in the depths of a solid medium *B. proteus* grows in discrete colonies. The swab or other material from which a culture is to be made is spread on blood-agar, and melted agar at about 45° C. is then poured over the surface of the plate to a depth of 2 or 3 mm. and allowed to set. After incubation it will be found that any colonies of *B. proteus* growing between the two layers of agar show no tendency to spread, and colonies of other organisms can be easily picked out from among them. It generally happens, however, that some of the *proteus* spreads round the edge of the agar on to the upper surface, and this must be killed before any deep colonies are picked out. A satisfactory method is by flooding the surface of the plate with saturated mercuric chloride solution for about thirty seconds. This is washed off with tap-water, and some of the surface growth of *proteus* is scraped off with the end of a microscope slide to enable the deep colonies to be examined. These can then be picked out and subcultured.

If the colonies are very crowded, and it is thought that the one picked out may be contaminated by a neighbouring colony of *B. proteus*, it is advisable to cover the subculture with a layer of agar in the same way. By this means, repeated more than once if necessary, it is possible to get a pure culture of most organisms, no matter how thick the contamination may be.

RESULTS.

Using the method described above, it has on every occasion been found possible to obtain pure cultures of *Streptococcus pyogenes* and of *Staphylococcus aureus* from mixtures of these organisms with *B. proteus*. This was mainly done with artificially mixed cultures, using twelve different strains of *B. proteus* and many strains of streptococcus and staphylococcus. The method has also always been successful when applied to naturally occurring mixed cultures. It may be noted here that it is particularly well adapted for the isolation of hæmolytic streptococci, as many strains which in primary culture show very weak hæmolysis on the surface of blood-agar give large, well-defined rings of hæmolysis in deep cultures.

With the anaërobic strains of streptococcus a few failures occurred at first, because of the difficulty of recognizing their deep colonies, which may be very small even after 48 hours' incubation. With a little experience in the appearance of these colonies, however, it is possible to pick them out with very little difficulty.

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THE PROPAGATION OF FUJINAMI'S FOWL MYXOSARCOMA IN DUCKLINGS.

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FUJINAMI'S observation that his fowl myxosarcoma grows equally well in some varieties of ducks and in fowls has been confirmed in a previous paper (Gye, 1931). The present communication deals with further experiments in which the tumour has been propagated in series in ducklings.

It had previously been found that the most satisfactory variety of duck available is that known as Khaki Campbell, and that young ducklings are more susceptible than half-grown or full-grown ducks. Hence the ducks used in all the experiments to be described were Khaki Campbells.

Fujinami's researches were directed to the discovery of a tumour which is transplantable in two distinct species of animals; he apparently neglected to distinguish sharply between the two processes by which tumours may be propagated, namely by transplantation and by infection. The Fujinami myxosarcoma is propagable in both fowls and ducklings, both by means of cell-free filtrates, *i. e.* by infection, and by means of cell transplantation (Gye, 1931). Whether propagation in ducks by cell transplants is or is not accompanied by transformation of normal duck cells to malignant cells by a process of infection will be dealt with by my colleague, Dr. Purdy, in later papers.

When ducklings younger than four weeks are injected with cell emulsions or with cell-free filtrates a tumour forms rapidly, and the duckling dies as a rule within ten days. The tumour is usually palpable on the fourth day after inoculation; it grows very rapidly, never metastasises, and has the structure of a spindle-cell sarcoma. In older ducks tumours appear almost always within a week after an injcetion of cells or filtrate, grow for a few days and then invariably disappear. The ease with which tumours can be induced in ducks and the certainty with which they disappear is a very puzzling phenomenon.

The dose of filtrate used in these experiments was usually 1·0 c.c., never more than 2·0 c.c. The volume of cell emulsion injected was always 0·05 c.c.

On March 21st, 1931, a routine Fujinami tumour in a fowl was taken and the tissue ground with sand; the broken-up tissue was extracted with saline; the extract was spun to deposit sand and tissue *débris*, and then filtered through a paper-pulp-sand filter. The filtrate was again filtered through paper-pulp and sand. The final filtrate was used for inoculating a duckling—No. 60—and two chickens, Nos. 69 and 70. The duckling received 0·5 c.c. in the right leg, the chickens 1 c.c. each in the right pectoral muscles. All three birds developed tumours. Chicken No. 70 was killed on April 7th, chicken 69 died on April 12th.

The duckling had a very large tumour in 8 days, and was killed on April 1st. The tumour was removed and used for the preparation of paper-pulp-sand filtrate, which was injected into the right leg—dose 1 c.c.—of duckling No. 61.

Again, on April 10th the tumour which had grown in duckling No. 61 was filtered and propagated by means of pulp-sand filtrate in ducklings Nos. 62 and 63.

Propagation was carried on from this point until May 22nd by means of cell grafts, when, a more abundant supply of ducklings having become available, it was decided to interpose in the series a transmission with a candle filtrate. During the two months between March 21st and May 22nd the tumour had been carried through seven generations in ducklings.

On May 22nd two tumours, from ducklings Nos. 91 and 93, were taken and an extract made, which was passed through a Berkefeld N candle. This filtrate was injected into three ducklings—Nos. 97, 98 and 99. It ought to be remarked here that candle filtrates of tumours are not always active either in ducklings or fowls. In order, therefore, to avoid the possible loss of the tumour which had already been passed through ducklings for seven generations, ducklings Nos. 94, 95 and 96 were injected at the same time with a cell emulsion of tumours taken from ducklings Nos. 91 and 93. In the present case, however, the candle filtrate proved to be very potent, and tumours appeared rapidly in all three of the ducklings 97, 98 and 99.

From the tumours thus obtained further propagations were carried on in ducklings, by injections of cell emulsions, until August 18th, 1931. By this time the tumour had been passed through eighteen generations of ducklings, which was regarded as sufficient to verify Fujinami's contention that his fowl myxosarcoma is propagable in series in ducks. The experiment was therefore stopped.

In all, 44 ducklings were used in this experiment ; 39 died of their tumours, or were killed when moribund for the purposes of the experiment ; the remaining 5 all developed tumours, which retrogressed.

Chickens were injected with cells or with filtrates of the tumour growing in ducklings of the second, seventh and eighteenth generations. In each case tumours formed. The tumours in chickens which followed injections of 1 c.c. doses of cell-free filtrate of the eighteenth generation of the tumour in ducklings were indistinguishable from routine Fujinami tumours in fowls.

Tumour tissue taken from the tenth and eleventh generation in ducklings was dried, and the dry powder was proved to be potent in both ducklings and chickens.

It has already been stated that half-grown or older ducks respond to injections of tissue or cell-free extract by the formation of tumours, but that these tumours invariably retrogress. Since a tumour which is formed as a result of the injection of cell-free extracts is necessarily a tumour of duck cells, and is therefore a true duck tumour, it might be expected that continued propagation of such a tumour in ducklings by cellular grafts would lead to the acquisition by the tumour-cells of the property of unlimited growth in adult ducks. The point may be stated by reference to well-known properties of mammalian tumours. When a sporadic tumour of a mouse is grafted in a large number of normal mice, the rule is that the graft grows in a small percentage of the mice

which are inoculated. By continuing the grafting from the best tumour of the series into another series of normal mice, and so on, eventually it is often possible to obtain a tumour which is transplantable in approximately 100% of normal mice and grows until the host is killed. The hope was entertained that the Fujinami tumour, after growth in a series of ducklings, might become adapted to continuous growth in adult ducks in the manner that transplants of malignant tissues usually grow. The hope, however, was disappointed. Half-grown and adult ducks were injected with cell emulsions made from the tenth, twelfth and fourteenth generations of duckling tumours. In each case tumours formed rapidly and then retrogressed. The result was no different from what is observed when fowl Fujinami tumour-cells are injected.

There is a fundamental difference between the growth of Fujinami tumours in fowls and in ducks. A tumour in a fowl, whether it is caused by the injection of a cell-free filtrate or whether it follows a cell graft, continues to grow and inevitably kills the host; the fowl cell is unable to resist the action of the virus. In ducks the virus is able to infect connective-tissue cells, which respond by very rapid growth—indeed more rapid than is ever shown by fowl cells—but unknown forces which resist the action of the virus come into play and lead to complete disappearance of the tumour, leaving the duck solidly immune to further injections of filtrates or cells. It is possible that the forces that enable duck cells to conquer the infective agent and thus to cure the animal of its tumour are the same as those which sometimes, though very rarely, lead to the disappearance of spontaneous tumours. If this should happen to be the case, then the study of Fujinami's tumour in ducks might afford an opportunity for the investigation of the natural forces which are directed towards the destruction of naturally occurring tumours.

SUMMARY.

Fujinami's fowl myxosarcoma has been propagated through eighteen generations in ducklings.

The infective agent retains its power to infect chickens.

Half-grown or older ducks form tumours in response to injections of cell emulsions, or of cell-free extracts, but the tumours retrogress.

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A MORPHOLOGICAL STUDY OF PSITTACOSIS VIRUS, WITH THE DESCRIPTION OF A DEVELOPMENTAL CYCLE.

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In the past the morphology of psittacosis virus has been studied almost entirely in preparations made from animals either dead or moribund from the disease. This is true at least of the work in this laboratory, where, during the past two years, a large number of preparations from the tissues of parrots, budgerigars, guinea-pigs and mice have been examined. And although the elementary bodies have shown occasionally some slight variation in size, their shape and appearance have presented a quite uniform picture, giving no indication whatever that the virus ever assumed any other form. Coles (1930) did indeed describe bacillary forms, but this has not been confirmed, and Levinthal (1931) was aware that the virus bodies varied somewhat in size—the name *Microbacterium multiforme psittacosis* suggested by him conveys this implication—but the literature contains no suggestion that the virus ever departed from the minute form in any stage of its development.

EXPERIMENTAL.

Recently some experiments were made by us with the object of learning more about the natural history of the psittacosis virus. We wished to find out where precisely in the animal body the virus multiplied, whether this always took place inside cells, the type of cell implicated, and at what period of disease the blood-stream became invaded. Mice were employed in this work, and the virus introduced by the intraperitoneal route. At intervals after inoculation mice were killed, the peritoneal exudate, heart's blood, spleen and liver examined quantitatively for virus, and at the same time a morphological study of the virus was made in stained preparations made from these different materials. The strain of virus used was one isolated from a parrot in 1930, which had been maintained since in mice, and had attained its 91st passage in that animal species. The first early material examined was from a mouse 24 hours after the intraperitoneal inoculation of 0·5 c.c. of a 5 per cent. suspension of virulent mouse spleen. Such a dose usually killed animals in 48 to 72 hours. The animal in question showed signs of commencing illness. Impression preparations made from the spleen and stained by Giemsa revealed a quite unexpected

picture. The clumps of virus, both intra- and extra-cellular, no longer consisted of uniform masses of elementary bodies, but of collections of elementary bodies, together with many much larger forms. These large forms were circular or oval, definite in shape and outline, and stained generally more faintly than the elementary bodies. The largest forms attained 1μ or more in size, and some showed quite clear evidence of division. Generally speaking there was a gradation in staining from the largest forms, which stained a pale blue purple, to the elementary bodies, which, as is usual in Giemsa preparations, appeared as deep purple bodies. A characteristic clump of virus from this preparation is depicted in Plate I, fig. 1, and Plate II, figs. 1 and 2, and a similar intracellular group is shown in Plate I, fig. 4. The regular, clean-cut appearance presented by these large forms, and the fact that they had never been met with in late material, argued against their being involution forms of elementary bodies. That they were not fragments of cell cytoplasm was clear, for when stained with formol-Borrel blue and counter-stained with safranin—a modification of Castaneda's stain—they stained blue like the elementary bodies and not red like cell cytoplasm. The suggestion that the virus underwent some developmental cycle was so strongly indicated by this finding that further work was undertaken to see if this were so or not. This has consisted of the examination of preparations made from spleen of mice killed at varying intervals after infection and the study of the virus in tissue culture.

Study of Developmental Forms in the Spleen of Infected Mice.

In this work large doses of a highly virulent virus have been employed. It was thought that in overwhelming infections running a rapid and fatal course, evidence of a developmental cycle, should one exist, would be more clearly obtained, for the shorter the illness the less chance there would be of more than one developmental cycle having taken place before death ensued. Thus at any given moment after infection one might expect to find the bulk of the virus at the same stage of development. The findings were clear-cut and bore out our expectations. Smears made from the spleens of mice which had died or were moribund showed the presence of innumerable elementary bodies, but apart from some slight variation in size of these particles, nothing else was noted. This bore out previous experience. Preparations made from the spleens of mice killed 24–30 hours after infection —that is, about halfway through their illness—invariably showed the presence of the larger forms to which reference has already been made. At this stage elementary bodies were present, but they only constituted about a third, or half of the particles; the remainder were larger forms. As had been noted previously, these larger forms varied in size from quite large bodies, about the size of a staphylococcus, down to the elementary bodies. Dividing forms were frequently encountered, and the gradation in staining from the largest forms, which took on a pale blue-purple, to the elementary bodies, which stained a deep blue purple, was in evidence. In such preparations, too, intracellular clumps were seen, which consisted of collections of deeply staining bodies intermediate in size between the largest free forms and the elementary bodies. Such a clump is illustrated in Plate I, fig. 5. At a still earlier stage in infection,

15 hours after inoculation, spleen impression preparations showed no elementary bodies. The virus was mostly in the form of large forms, which again showed evidence of division. These forms were seen inside large mononuclear cells and also free, probably as the result of rupture of an infected cell. They are illustrated in Plate I, figs. 2, 3 and 6, and in Plate II, figs. 3 and 4, and it must be emphasized here again that when stained by Castaneda's method they behave like the elementary bodies in that they retain the blue (Plate I, fig. 3). In similar preparations homogeneous plaques of material which stained like virus were observed both inside and outside cells (Plate I, figs. 8 and 9; Plate II, fig. 5). Occasional plaques were met with, however, in which the early stage of division of the plaque into the large virus forms was seen (Plate I, fig. 7). Preparations made from still earlier spleens, removed 5 to 6 hours after infection, showed only undivided plaques, and these few and small. In every case a portion of spleen was rubbed on an agar slope and another portion dropped into a broth tube. No growth was observed during 7 days' incubation at 37° C.

Study of Developmental Forms in Tissue Culture.

The tissue used in these experiments was mouse spleen, cultivation being carried out on coverslips in mouse serum, diluted with equal parts of either Compton-Pannett saline or chick embryo extract. Three experiments were made. In two of these the splenic tissue was obtained from infected mice 5 hours after inoculation ; in the third, fragments of normal mouse spleen were infected by soaking them in a psittacosis filtrate for an hour in the refrigerator. Cultures have been incubated at 37° and 32° C., the latter temperature having been shown by Pinkerton and Haas (1932) to be much superior to 37° C. for the study of *rickettsia* in culture. From time to time after setting up the experiment, cultures were withdrawn from the incubator and fixed and stained for virus. Both Giemsa and Castaneda's stain were used. In one experiment a parallel series of cultures, made, however, with larger fragments of tissue, was used for virus titrations in the guinea-pig's skin ; the result showed a 1000-fold increase of virus in 72 hours. It will be unnecessary to describe the findings in these experiments in detail ; suffice it to say that they bore out completely the observations made in the *in vivo* experiments. The virus was seen to pass through the same sequence of morphological changes, though cultures incubated at 32° C.—which incidentally gave us the best pictures—took rather longer to reach the stage of elementary bodies than was the case in the animal. Stages in the development of the virus obtained in tissue culture are illustrated in Plate I, figs. 10, 11 and 12. In tissue culture no virus was seen outside the cells ; it seems to require this situation for its development.

INTERPRETATION OF THE FINDINGS.

In our opinion the findings which we have obtained in both types of experiment leave no doubt that the virus of psittacosis is not always in the form of elementary bodies, but passes through a developmental cycle, of which the elementary body is the last phase. It must be emphasized that not only are

EXPLANATION OF PLATES.

Plate I.

Fig. 1.—Group of virus lying free in an impression preparation made from the spleen of a mouse removed 24 hours after infection. Giemsa differentiated in orange G-tannin. × 1500. Both elementary bodies and larger forms present. Large forms appear to be dividing and intermediate forms occur. Note gradation in staining.

Fig. 2.—Group of virus lying free in an impression preparation made from the spleen of a mouse removed 15 hours after infection. Giemsa differentiated in orange G-tannin. × 1500. Note the irregularity in the shape of the large particles, unlike what one would expect were they merely large forms of a coccus.

Fig. 3.—Virus at same stage of development as in Fig. 2, but inside a cell. (Morula) Impression preparation of mouse spleen removed 15 hours after infection. Castaneda. × 1500. Note that the large virus forms, like elementary bodies, retain the blue stain. Cell cytoplasm and chromatin take the safranin used as counterstain.

Fig. 4.—Virus in same stage of development as in Fig. 1, but intracellular. Impression preparation of mouse spleen removed 24 hours after infection. Giemsa differentiated in orange G-tannin. × 1200.

Fig. 5.—Intracellular mass of virus intermediate in stage between morula and elementary bodies. Impression preparation of mouse spleen removed 24 hours after infection. Giemsa differentiated in orange G-tannin. × 1200.

Fig. 6.—Small morula inside a cell. Impression preparation of a mouse spleen removed 24 hours after infection. Giemsa. × 1200.

Fig. 7.—Plaque (plasmodium) in which there are indications of commencing division. Smear of mouse spleen removed 15 hours after inoculation. Castaneda. × 1200.

Fig. 8.—Plaque (plasmodium) which appears homogeneous. Impression preparation of mouse spleen removed 15 hours after inoculation. Giemsa differentiated in orange G-tannin. × 1200. The plaque is stained a dirty greenish blue.

Fig. 9.—Plaque (plasmodium) in same stage as in Fig. 8. Impression preparation of mouse spleen removed 15 hours after inoculation. Castaneda. × 1200. The plaque is stained blue, differentiating it from cytoplasm or nuclear material, which take the safranin by this staining method.

Fig. 10.—Plaque (plasmodium) just beginning to divide. Tissue culture made from normal mouse spleen soaked in virus filtrate and grown in mouse serum and chick embryo extract. 24 hours' incubation at 32° C. Fixation: Schaudinn. Giemsa. × 1200.

Fig. 11.—Cell showing three masses of virus in morula stage. Tissue culture. From same experiment as Fig. 10, but after 48 hours' incubation at 32° C. Fixation: Schaudinn. Giemsa. × 1200.

Fig. 12.—Cell showing mass of elementary bodies. Tissue culture. From same experiment as Figs. 10 and 11, but after 4 days' incubation at 32° C. Fixation: Schaudinn. Giemsa. × 1200.

Plate II.

Fig. 1.—Free clump of virus in impression preparation of mouse spleen removed 24 hours after inoculation. Shows variation in size, the smallest particles being elementary bodies. Giemsa. × 900.

Fig. 2.—Free clump of virus in impression preparation of mouse spleen removed 15 hours after inoculation. Mostly large forms, which are often in pairs, suggesting division. Giemsa. × 900.

Fig. 3.—Disrupted morula in impression preparation of mouse spleen removed 15 hours after inoculation. Shows irregularity in size and shape of these forms. Giemsa. × 900.

Fig. 4.—Morula in impression preparation of mouse spleen removed 15 hours after inoculation. Giemsa. × 900.

Fig. 5.—Virus plaque in impression preparation of mouse spleen removed 15 hours after inoculation. Photograph does not show the cell cytoplasm in which the plaque was lying. Giemsa. × 900.

Fig. 6.—Virus mass which has reached the stage of elementary bodies in impression preparation from mouse spleen removed 3 days after inoculation. The virus is intracellular, but the cell cytoplasm does not appear in the photograph. Giemsa. × 900.

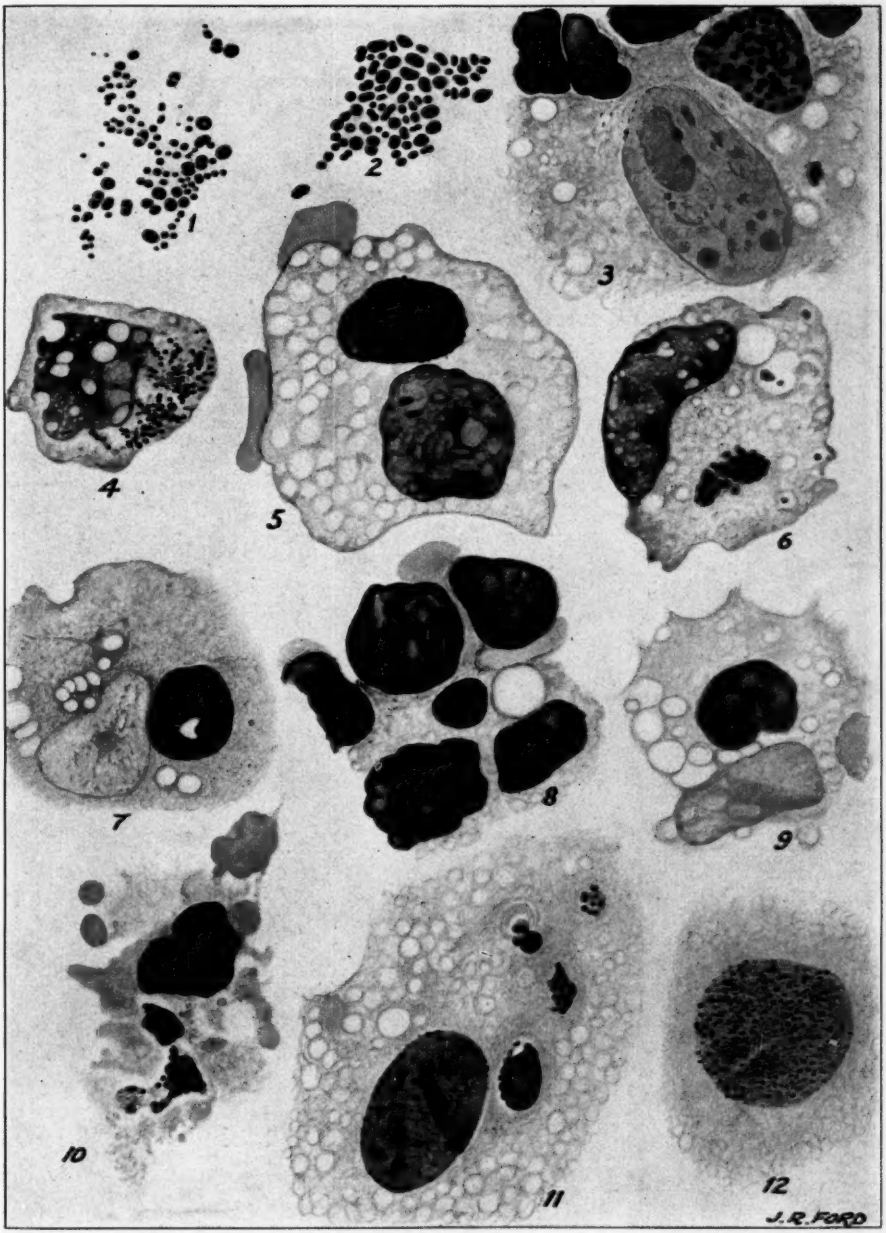

Plate I. Bedson and Bland.

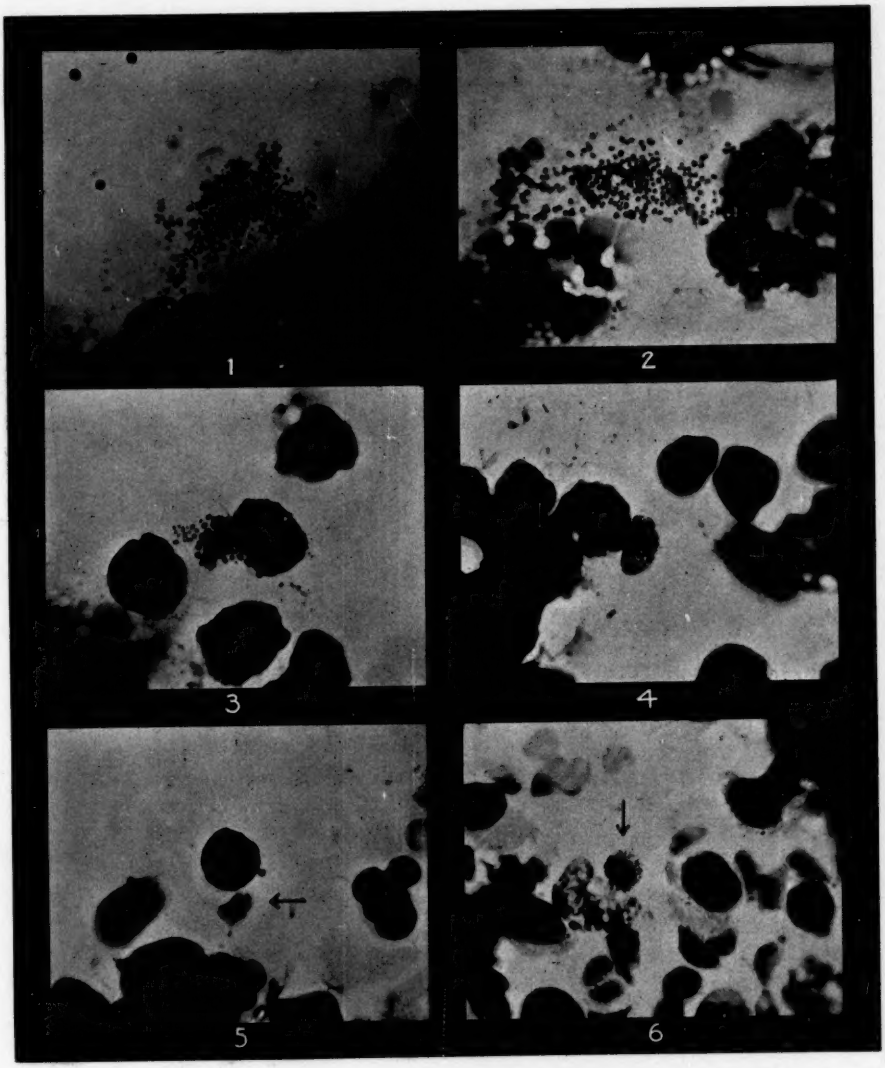

Plate II.

Bedson and Bland.

these various stages quite evidently linked in morphological sequence, but also in time. Given a massive infection with a potent virus, the different stages can be obtained more or less at will by simply killing the animal at the required time. There are two possible explanations of the cycle of changes noted. One is that the elementary bodies, having reached a suitable environment in the host's tissues—in our opinion the large mononuclear phagocytic cells (of the spleen in these experiments)—proceed to multiply, and in the early stages of this multiplication give rise to the large forms, such as are illustrated in Plate I, fig. 2, and Plate II, fig. 3. These, in turn, divide, and as the cycle of development progresses, the form assumed by the virus becomes progressively smaller, until particles the size of the elementary bodies are again reached. The plaque forms in such a cycle as this could only be explained on the supposition that they were virus colonies surrounded by a sheath of homogeneous material which prevented the virus particles being seen. In other words the plaque would be an inclusion body, but in this case a basophile one, and unlike the other cytoplasmic virus inclusions which, of course, are acidophile. The other explanation of the sequence of events, and the one to which we very definitely incline, would be as follows: The elementary bodies, having gained susceptible cells, develop into amœboid forms, which coalesce to form a plasmodium. Plaques of varying size and shape may be seen, and cells occur containing more than one virus plaque which has reached the stage of commencing division (Plate I, fig. 11). It would seem unreasonable to suppose that one elementary body could give rise to a huge plaque occupying the major portion of a cell, and the evidence suggests that the larger plaques at any rate are the result of fusion of smaller ones. According to this hypothesis the plaque would be a plasmodium. This plasmodium then divides up into a number of portions of equal size, roughly oval in shape, and a phase is thus developed in which the virus is revealed as a spherical body packed with oval segments (Plate I, figs. 3, 6, 11; Plate II, fig. 4)—what might be termed a morula in fact. These oval segments then divide and subdivide, showing increasingly basophile staining as the particles become smaller. The result is the production of elementary bodies (Plate I, fig. 12; Plate II, fig. 6), but whether these are the end-product of division, or whether they arise inside the small divisional forms by a process of condensation like a small spore, we are not prepared to say. Such a cycle—elementary body → amœboid forms running together to form a plasmodium → morula → division and subdivision of elements of morula → elementary body—carries with it the implication that the virus is not a bacterium, but possesses affinities with the mycetozoa or myxomycetes, or possibly with the microsporidia. In this connection it is of interest to recall that Levaditi, Nicolau and Schoen (1926) suggested that the virus of rabies resembled a microsporidian. There are several reasons which incline us strongly to accept this second explanation of the developmental cycle, even though it carries with it a rather unorthodox implication. It seems difficult to believe that the plaques are inclusion bodies surrounded by a wall through which it is impossible to see, for from the morula stage onwards every detail of the virus colonies can be clearly resolved. Why should this sheath only exist in the earliest stages and disappear so soon? Further, the plaque has the staining affinities of the virus, and not those of a typical inclusion body. Then, again, the large forms

which constitute the morula, when freed by disruption of the cells, are not regularly oval or round, as one would expect if they were merely large coccal forms. They have flattened sides and are often not quite regular in shape, just as one might expect had they arisen by division of a plaque of material. For these reasons we conclude that the plaques which we have seen represent a plasmodial phase of the virus.

As far as can be ascertained, this cycle takes some 48–72 hours in the mouse, though in tissue culture at 32° C. it takes longer—up to 4 or 5 days. Whether the virus is infective in all stages or only in the form of elementary bodies we do not know as yet, though suspensions of spleens in which only large forms could be seen have produced reactions in the guinea-pig's skin. This is a point which it is proposed to investigate carefully, and the possibility of using the filter and centrifuge to gain confirmation of this morphological study suggests itself. Apart from the question of the nature of psittacosis virus which this work suggests, and which has already been discussed, there is another which arises. Is psittacosis virus unique among the filterable viruses, or do others pass through a similar developmental cycle? Reference has already been made to the view expressed by Levaditi, Nicolau and Schoen (1926) that rabies virus presents analogies with the microsporidia, but clearly direct observation alone can provide the answer to this question. In this connection, however, it is interesting to recall that doubt has been expressed at times about the possibility of minute particles like elementary bodies—at any rate the smaller ones—multiplying by division. In such a cycle as the one we postulate for psittacosis virus this difficulty does not arise, for the elementary bodies emanate from larger forms.

CONCLUSIONS.

1. The virus of psittacosis occurs in forms other than elementary bodies.
2. The appearance of these forms and their relation to the different stages of infection indicate that the virus passes through a developmental cycle.
3. The significance of these observations is discussed.

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